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(54) Title: ADENOVIRAL VECTORS FOR TREATMENT OF HEMOPHILIA

(57) Abstract

An adenoviral vector including at least one DNA sequence encoding a clotting factor, such as, for example, Factor VIII, or Factor IX. Such vectors may be administered to a host in an amount effective to treat hemophilia in the host. The vectors infect hepatocytes very efficiently, whereby the hepatocytes express the DNA sequence encoding the clotting factor.

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ADENOVIRAL VECTORS FOR TREATMENT OF HEMOPHILIA

This application is a continuation-in-part of application Serial No. 074,920, filed June 10, 1993.

Field of the Invention

This invention relates to adenoviral vectors. More particularly, this invention relates to adenoviral vectors which may be employed in the treatment of hemophilia.

Background of the Invention

Hemophilias A and B are X-linked, recessive bleeding disorders caused by deficiencies of clotting Factors VIII and IX, respectively. In the United States there are approximately 17,000 patients with hemophilia A and 2,800 with hemophilia B. The clinical presentations for both hemophilias are characterized by episodes of spontaneous and prolonged bleeding. Patients frequently suffer joint bleeds which lead to a disabling arthropathy. Current treatment is directed at stopping the bleeding episodes with intravenous infusions of plasma-derived clotting factors or, for hemophilia A, recombinant Factor VIII. However, therapy is limited by the availability of clotting factors, their short half-lives *in vivo*, and the high cost of treatment, which can approach 100,000 dollars per year.

Gene therapy offers the promise of a new method of treating hemophilia. Several groups of researchers have conducted

research with retroviral vectors containing RNA encoding Factor VIII and Factor IX. Virtually every attempt to date to produce therapeutic levels of these factors in vivo with such vectors, however, has been unsuccessful. The cDNA and the RNA for Factor VIII has been particularly difficult to work with.

Hoeben, et al., J. Biol. Chem, Vol. 265, pgs 7318-7323 (1990) and Israel, et al. Blood, Vol. 75, No. 5, pgs. 1074-1080 (March 1, 1990) describe the infection of mouse fibroblasts in vitro with retroviral vectors including DNA (RNA) encoding B-domain deleted human Factor VIII. Although such infected cells were found to express functional human Factor VIII *in vitro*, the protein was expressed at low levels.

Recently, Hoeben, et al., Human Gene Therapy, Vol. 4, pgs 179-186 (1993) infected fibroblasts with retroviral vectors including DNA encoding human Factor VIII. These cells then were implanted into immune-deficient mice. Although cells recovered from the implants up to 2 months post-implantation still had the capacity to secrete Factor VIII when regrown in tissue culture, human Factor VIII was not detected in plasma samples of the recipient mice.

Lynch, et al., Human Gene Therapy, Vol. 4, pgs. 259-272 (1993), describes the transfection of PE501 packaging cells with the plasmid forms of retroviral vectors including human Factor VIII cDNA. The virus was harvested, and used to infect PA317 amphotropic retrovirus packaging cells. The infected cells, however, produced human Factor VIII and virus titer in an amount which was about two orders of magnitude lower than those from similar retroviral vectors containing other cDNAs. Lynch, et al. also observed a 100-fold lower accumulation of vector RNAs containing the human Factor VIII sequences in comparison to vectors containing other cDNA sequences.

Lynch, et al. also reported the following difficulties in working with Factor VIII. High titer human Factor VIII-containing retroviral vector stocks are difficult to generate, and retroviral vectors containing Factor VIII cDNA sequences tend

to rearrange and/or delete portions of the Factor VIII cDNA sequences. In addition, Factor VIII mRNA is inherently unstable. Also, the B-domain deleted Factor-VIII coding region contains a 1.2 kb RNA accumulation inhibitory signal.

Thus, the literature discloses that there have been significant problems in working with retroviral approaches to gene therapy with Factor VIII and that only limited expression has been achieved. The applicants are not aware of any published reports in which human Factor VIII has been expressed in an animal model.

Researchers also have experienced significant difficulties in attempting to achieve therapeutic levels of Factor IX expression with retroviral vectors.

Palmer, et al., Blood, Vol. 73, No. 2, pgs. 438-445 (February 1989) discloses the transduction of human skin fibroblasts with retroviral vectors including DNA (RNA) encoding human Factor IX. Such transformed fibroblasts then were given to rats and to nude mice. Although such fibroblasts were found to transiently express human Factor IX in the animal blood in amounts up to 190 ng/ml, this amount is not generally considered to be at a therapeutic level.

Scharfmann, et al., Proc. Nat. Acad. Sci., Vol. 88, pgs. 4626-4630 (June 1991) discloses the transduction of mouse fibroblast implants with a retroviral vector including a B-galactosidase gene under the control of the dihydrofolate reductase (DHFR) promoter. Such fibroblasts then were grafted into mice, and expression of the β-galactosidase gene for up to sixty days was obtained. Scharfmann, et al. also disclose fibroblasts transduced with canine Factor IX, but they only obtained short-term and non-therapeutic levels of expression.

Dai, et al., Proc. Nat. Acad. Sci., Vol. 89, pgs. 10892-10895 (November 1992) discloses the transfection of mouse primary myoblasts with retroviral vectors including canine Factor IX DNA under the control of a mouse muscle creatine kinase enhancer and a human cytomegalovirus promoter. The transfected myoblasts then

were injected into the hind legs of mice. Expression of canine Factor IX over a period of 6 months was obtained; however, the steady-state levels of Factor IX secreted into the plasma (10 ng/ml for 10^7 injected cells) are not sufficient to be of therapeutic value.

Gerrard, et al., Nature Genetics, Vol. 3, pgs. 180-183 (February 1993), discloses the transfection of primary human keratinocytes with a retroviral vector including a human Factor IX gene under the control of the retroviral LTR. The transformed keratinocytes then were transplanted into nude mice, and human Factor IX was detected in the bloodstream for about 1 week. The amounts of Factor IX, however, were about 2.5 ng/ml, or about 1% of a therapeutic dose.

Kay, et al., Science, Vol. 262, pgs. 117-119 (October 1, 1993) discloses the direct infusion of retroviral vectors including Factor IX DNA into the portal vasculature of dogs following partial hepatectomy. The animals expressed low levels of canine Factor IX for more than 5 months. Although such expression of Factor IX resulted in reductions of whole blood clotting and partial thromboplastin times of the treated animals, the authors stated that increased levels of Factor IX must first be achieved before the technique could be applied to humans.

Zhou, et al., Science in China, Vol. 36, No. 9, pgs. 33-41 (September 1993) discloses the transfection of rabbit skin fibroblasts with retroviral vectors including DNA encoding human Factor IX. The fibroblasts then were implanted into rabbits as autografts or allografts. Expression of the human Factor IX was maintained in the rabbits for over 10 months. Factor IX levels in the rabbit plasma of up to 480 ng/ml were claimed to have been achieved; however, the assay used to measure Factor IX employed an anti-rabbit antibody that had the potential of generating false positive results.

Lu, et al., Science in China, Vol. 36, No. 11, pgs. 1341-1351 (November 1993) and Hsueh, et al., Human Gene Therapy, Vol. 3, pgs. 543-552 (1992) discloses a human gene therapy trial in

which human skin fibroblasts were taken from two hemophiliac patients, and transfected with retroviral vectors including DNA encoding human Factor IX. The cells then were pooled and embedded in a collagen mixture, and the cells then were injected into the patients. In one patient, the concentration of human Factor IX increased from 71 ng/ml to 220 ng/ml, with a maximum level of 245 ng/ml. The clotting activity of this patient increased from 2.9% to 6.3% of normal. In the other patient, the plasma level of Factor IX increased from 130 ng/ml to 250 ng/ml, and was maintained at a level of 220 ng/ml for 5½ months; however, the clotting activity has not increased. Lack of pretreatment Factor IX data on these patients makes it difficult to interpret the small increases in Factor IX seen in treatment.

The conclusion to be drawn from scientific literature on the attempts to use retroviruses in gene therapy for hemophilia A and hemophilia B is that, in spite of a very concerted effort and numerous attempts, by and large the field has failed to produce retroviral vectors that can be used to achieve therapeutic levels of expression of human Factor VIII or human Factor IX in vivo. Working with Factor VIII has been especially difficult, and the results have been uniformly unsatisfactory. The experimental strategies described above are laborious and clinically invasive.

Adenoviral vectors offer another approach to gene therapy. Adenovirus genomes are linear, double-stranded DNA molecules of approximately 36 kilobase pairs. Each extremity of the viral genome has a short sequence known as the inverted terminal repeat (or ITR), which is necessary for viral replication. The well-characterized molecular genetics of adenovirus render it an advantageous vector for gene transfer. Portions of the viral genome can be substituted with DNA of foreign origin. In addition, recombinant adenoviruses are structurally stable and no rearranged viruses are observed after extensive amplification.

Recombinant adenoviruses have been used as efficient vectors for gene transfer into a number of cell types. There are several reports of hepatocyte transduction: Jaffe, et al., Nature

Genetics, Vol. 1, pgs. 372-378 (1992) (alpha-1-antitrypsin); Li, et al., Human Gene Therapy, Vol. 4, pgs. 403-409 (1993) (beta-galactosidase); Stratford-Perricaudet, et al., Human Gene Therapy, Vol. 1, pgs. 241-256 (1990) (ornithine transcarbamylase); Smith, et al., Nature Genetics, Vol. 5, pgs. 397-402 (1993) (Factor IX); and J. Am. Med. Assoc., Vol. 269, No. 7, pg. 838 (February 17, 1993) (marker protein).

Because Factor VIII is synthesized largely in hepatocytes (Kelly, et al. Br. J. Haemat., Vol. 56, pgs. 535-543 (1984); Wion, et al., Nature, Vol. 317, pgs. 726-729 (1985); Zelechovska, et al. Nature, Vol. 317, pgs. 729-732 (1985)), transduction of hepatocytes with a Factor VIII - containing recombinant adenovirus, resulting in the expression of Factor VIII protein *in vivo*, may be an effective gene therapy-based treatment for hemophilia A.

The inventors have discovered how to produce high titer, stable, adenoviral vectors that produce therapeutic levels of clotting factors when administered to an animal host. These vectors mediate gene transfer in vivo and will enable treatment protocols to be much less laborious and invasive than those previously described.

Brief Description of the Drawings

The invention will now be described with respect to the drawings, wherein:

Figure 1 is a schematic of the construction of plasmid pG1.

Figure 2 is the sequence of the multiple cloning site in the pG1 plasmid;

Figure 3 is a map of plasmid pG1;

Figure 4 is a map of plasmid pG1H9;

Figure 5 is a map of plasmid phfacIX;

Figure 6 is a map of plasmid pG1H9B;

Figure 7 is a schematic of the construction of plasmid pHR;

Figure 8 is a schematic of the assembly of an ITR, encapsidation signal, Rous Sarcoma Virus promoter, adenoviral tripartite leader sequence, and a linking sequence using PCR amplification;

Figure 9 is a schematic of the construction of pAvS6;

Figure 10 is a map of pAvS6;

Figure 11 is the human Factor IX cDNA sequence;

Figure 12 is a map of pAvS6H9B;

Figure 13 is a schematic of the generation of Av1H9B;

Figure 14 is a graph of plasma levels of Factor IX in mice given intraparenchymal or portal vein injections of Av1H9B;

Figure 15 is an autoradiogram of a Southern analysis to determine the presence of Factor IX DNA in mouse liver;

Figure 16 is a map of plasmid pMT2LA;

Figure 17 is the sequence of B-domain deleted human Factor VIII cDNA;

Figure 18 is a map of plasmid pAvS6H81;

Figure 19 is a schematic of the construction of Av1H81;

Figure 20 is a map of plasmid pAT2-3eG.

Figure 21 is a map of plasmid pAvAL1;

Figure 22 is a map of plasmid pGEM(sac);

Figure 23 is a map of plasmid pGEM;

Figure 24 is a map of plasmid pGEMalb;

Figure 25 is a map of plasmid pGEMalbF8B;

Figure 26 is a map of plasmid pAvALH81;

Figure 27 is a map of plasmid pgemF8B2;

Figure 28 is a map of plasmid pBGS19-AIgI;

Figure 29 is a map of plasmid pUC19;

Figure 30 is a map of plasmid pUC19-AIgI;

Figure 31 is a map of plasmid pBGS19;

Figure 32 is a map of plasmid pGemAPF8B;

Figure 33 is a map of plasmid pAvAPH81;

Figure 34 is a map of plasmid pGemAPexF8;

Figure 35 is a map of plasmid pALAPF8B;

Figure 36 is a map of plasmid pAvALAPH81;

Figure 37 is a schematic of the generation of Av1ALH81;

Figure 38 is a blot of a restriction digestion analysis of Av1ALH81 DNA;

Figure 39 is a schematic of adenoviral vectors Ad5-dl327, Av1ALH81, and Av1ALAPH81;

Figure 40 is a standard log-log curve of a human Factor VIII - specific ELISA assay;

Figures 41 and 42 are graphs of the amounts of human Factor VIII in mouse plasma over time in two separate experiments;

Figure 43 is a graph of in vivo expression of human Factor VIII in mouse plasma over time after injection of various doses of Av1ALH81;

Figures 44 and 45 are graphs of human Factor VIII half-life in experimental mice and control mice;

Figure 46 is a graph of in vivo expression of human Factor VIII in mice given 4×10^9 pfu of Av1ALH81;

Figure 47 is a map of plasmid pBLSKH9CI;

Figure 48 is a map of plasmid pBLSKH9D;

Figure 49 is a map of Plasmid pBLH9CINT;

Figure 50 is a map of plasmid pBLH9EINT;

Figure 51 is a map of plasmid pBLH9E;

Figure 52 is a map of plasmid pBLH9F:

Figure 53 is a map of plasmid pAV1H9D;

Figure 54 is a map of plasmid pAV1H9ER;

Figure 55 is a map of plasmid pAV1H9FR;

Figure 56 is a graph of Factor IX expression in mice treated with Av1H9B, Ad1H9D, Ad1H9ER and Ad1H9FR;

Figure 57 is a graph of Factor IX expression in mice treated with 1×10^9 pfu of Av1H9B, Ad1H9D, or Ad1H9ER;

Figure 58 is a graph of Factor IX expression in mice treated with 5×10^7 pfu of Ad1H9ER or Ad1H9FR; and

Figure 59 is a graph of in vitro expression of Factor IX in HepG2 and HeLa cells, and of in vivo expression of Factor IX in mice treated with 2×10^8 pfu of Av1H9B, Ad1H9D, or Ad1H9ER. In

each group of 3 bars, the leftmost bar represents data for Av1H9B, the middle bar, Ad1H9D, and the rightmost bar, Ad1H9ER.

Detailed Description of the Invention

In accordance with an aspect of the present invention, there is provided an adenoviral vector including at least one DNA sequence encoding a clotting factor.

The term "DNA sequence encoding a clotting factor" as used herein means DNA which encodes a full-length clotting factor or a fragment, derivative, or analogue of a clotting factor, i.e., such DNA may be a full-length gene encoding a full-length clotting factor, or a truncated gene, or a mutated gene encoding a fragment or derivative or analogue of such clotting factor which has clotting factor activity. The term "DNA sequence" refers generally to a polydeoxyribonucleotide molecule and more specifically to a linear series of deoxyribonucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of the adjacent pentoses.

In one embodiment, the DNA sequence encodes Factor VIII or a fragment, derivative, or analogue thereof having Factor VIII clotting activity. In another embodiment, the DNA sequence encodes Factor IX or a fragment, derivative, or analogue thereof having Factor IX clotting activity.

The DNA sequence encoding human Factor IX is shown and described in U.S. Patent No. 4,994,371 issued February 19, 1991 to Davie, et al. and European Patent No. EP 0 107 278 B1 (publication of grant November 15, 1989) to National Research Development Corporation. DNA sequences encoding Factor VIII and fragments or derivatives thereof are shown and described in U.S. Patent Nos. 4,757,006 issued July 12, 1988 to Toole, Jr. et al.; 4,868,112 issued September 19, 1989 to Toole, Jr.; 5,045,455 issued September 3, 1991 to Kuo, et al; 5,004,804 issued April 2, 1991 to Kuo, et al.; 5,112,950 issued May 12, 1992 to Meulien, et al.; and 5,149,637 issued September 22, 1992 to Scandella, et al.

The inventors have found that, by infecting host cells in vivo with adenoviral vectors including at least one DNA sequence encoding a clotting factor, one is able to achieve expression, in vivo, of the clotting factor, or fragment or derivative or analogue of such clotting factor having clotting factor activity, at effective therapeutic levels. In general, such effective therapeutic levels are about 5% or greater of the normal level of the clotting factor (N. Engl. J. Med., Vol. 328, No. 7, pgs. 453-459 (February 18, 1993); Blood, Vol. 74, No. 1, pgs. 207-212 (July 1989)). Such levels are, in general, for Factor VIII, about 10 ng/ml or greater, and for Factor IX are about 250 ng/ml or greater.

The DNA sequence encoding a clotting factor is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus promoter; the Rous Sarcoma Virus (RSV) promoter; the albumin promoter; inducible promoters, such as the Mouse Mammary Tumor Virus (MMTV) promoter; the metallothionein promoter; heat shock promoters; the α-1-antitrypsin promoter; the hepatitis B surface antigen promoter; the transferrin promoter; the apolipoprotein A-1 promoter; the Factor VIII promoter; and the Factor IX promoter. It is to be understood, however, that the scope of the present invention is not to be limited to specific promoters.

In one embodiment, when the DNA sequence encodes Factor VIII or a fragment, derivative, or analogue thereof, the promoter controlling the DNA sequence is preferably a tissue-specific promoter, such as, for example, the mouse albumin promoter, which is active in liver cells. Although the scope of this embodiment is not intended to be limited to any theoretical reasoning, the inventors believe that, by employing a tissue-specific promoter, possible Factor VIII toxicity to the producer cells is avoided.

When one employs a mouse albumin promoter, which is active

in liver cells, the adenoviral vectors are preferably grown in cells other than liver cells. When the generated adenoviral vectors are to be administered to a host, such vectors are administered to a host by means known to those skilled in the art, whereby the vectors will travel to and infect liver cells. The infected liver cells then will express Factor VIII in therapeutic amounts. Factor VIII is not toxic to liver cells and thus will continue to be expressed at therapeutic levels.

In yet another embodiment, when the DNA sequence encodes Factor IX or a fragment, derivative, or analogue thereof, the promoter controlling the DNA sequence is preferably a strong promoter that is not tissue-specific, such as, for example, the Rous Sarcoma Virus promoter. Because it is believed that Factor IX is not toxic to most cells, the adenoviral vectors may be grown in any cell type, and may be administered to a patient in an effective therapeutic amount, whereby the adenoviral vectors will travel to and infect cells such as liver cells, for example, whereby the Factor IX will be expressed in therapeutic amounts.

Several reports have revealed that, in transgenic mice, enhanced expression of cDNA's can be obtained by the incorporation of 5' and 3' untranslated regions as well as introns (Choo, et al. Nucl. Acids Res., Vol. 15, pgs. 881-884 (1987); Brinster, et al. PNAS, Vol. 85, pgs 836-840 (1988); Jallat, et al. EMBO J., Vol. 9, No. 10, pgs. 3295-3301 (1990); and Choi, et al. Mol. Cell. Biol., Vol. 11, pgs. 3070-3074 (1991)). The effectiveness, however, of genomic elements in improving expression of exogenous genes incorporated into an adenoviral vector backbone has not been demonstrated previously.

In one embodiment, the DNA sequence encoding a clotting factor also may include introns and other genomic elements to enhance expression. The term "genomic element," as used herein, means a sequence of nucleotides in a naturally occurring gene that is not normally incorporated into the cDNA, and which is not part of the adenoviral genome. Such genomic elements which may be included in the vector include, but are not limited to,

introns, the 5' untranslated region, and the 3' untranslated region of the gene encoding the clotting factor, or portions of such 5' and 3' untranslated regions and introns. Examples of introns which may be employed include, but are not limited to, any of the seven introns of the Factor IX gene, or portions thereof (EMBO J., Vol. 9, No. 10, pgs. 3295-3301 (1990)); or any of the twenty-five introns of the Factor VIII gene (Gitschier, Nature, 312:326-330 (1984)), or portions thereof; or the first exon and intron of the apolipoprotein A-1 gene.

When the DNA sequence encodes Factor IX or a fragment, derivative, or analogue thereof, the vector may, in one embodiment, further include the full 3' untranslated region of the Factor IX DNA sequence. In another embodiment, the vector may further include the full 5' untranslated region and a centrally truncated first intron. In yet another embodiment, the vector may further include the full 3' untranslated region, the full 5' untranslated region, and a centrally truncated first intron. Most preferably, the vector contains all of these elements. In a further embodiment, the vector may further include the full 7th intron of the Factor IX gene.

When such elements are included in the vector, improved levels of expression of Factor IX are obtained. Although the scope of the present invention is not intended to be limited to any theoretical reasoning, such improved expression may be due to (i) the incorporation of enhancers in the genomic sequences; (ii) stabilization of the mRNA; (iii) improved processing and transport of the mRNA to the cytoplasm; and/or (iv) improved polyadenylation.

In another embodiment, the first exon and first intron of the apolipoprotein protein A-1 gene may be employed, if desired, with the apolipoprotein A-1 gene promoter. (PNAS, Vol. 80, pgs. 6147-6151 (October 1983); J. Biol. Chem., Vol. 266, No. 27, pgs. 18045-18050 (Sept. 1991)). The above-mentioned introns and/or exons also may be used in combination with the 5' untranslated

region and/or the 3' untranslated region of the gene encoding the clotting factor.

In one preferred embodiment, the apolipoprotein A-1 promoter may be employed, alone or in combination with the first exon and/or first intron of the apolipoprotein A-1 gene, in combination with the Factor VIII gene.

The adenoviral vector which is employed may, in one embodiment, be an adenoviral vector which includes essentially the complete adenoviral genome (Shenk, et al., Curr. Top. Microbiol. Immunol., 111(3): 1-39 (1984). Alternatively, the adenoviral vector may be a modified adenoviral vector in which at least a portion of the adenoviral genome has been deleted.

In the preferred embodiment, the adenoviral vector comprises an adenoviral 5' ITR; an adenoviral 3' ITR; an adenoviral encapsidation signal; at least one DNA sequence encoding a clotting factor; and a promoter controlling the at least one DNA sequence encoding a clotting factor. The vector is free of at least the majority of adenoviral E1 and E3 DNA sequences, but is not free of all of the E2 and E4 DNA sequences, and DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter. In one embodiment, the vector is also free of at least a portion of at least one DNA sequence selected from the group consisting of the E2 and E4 DNA sequences.

In another embodiment, the vector is free of at least the majority of the adenoviral E1 and E3 DNA sequences, and is free of one of the E2 and E4 DNA sequences, and is free of a portion of the other of the E2 and E4 DNA sequences.

In still another embodiment, the gene in the E2a region that encodes the 72 kilodalton binding protein is mutated to produce a temperature sensitive protein that is active at 32°C, the temperature at which the viral particles are produced, but is inactive at 37°C, the temperature of the animal or human host. This temperature sensitive mutant is described in Ensinger, et al., J. Virology, 10:328-339 (1972), Van der Vliet, et al., J.

Virology, 15:348-354 (1975), and Friefeld, et al., Virology, 124:380-389 (1983).

In yet another embodiment, the vector is free of at least the majority of the E1 and E3 DNA sequences, is free of at least a portion of at least one DNA sequence selected from the group consisting of the E2 and E4 DNA sequences, and is free of DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter.

Such a vector, in a preferred embodiment, is constructed first by constructing, according to standard techniques, a shuttle plasmid which contains, beginning at the 5' end, the "critical left end elements," which include an adenoviral 5' ITR, an adenoviral encapsidation signal, and an E1a enhancer sequence; a promoter (which may be an adenoviral promoter or a foreign promoter); a multiple cloning site (which may be as hereinabove described); a poly A signal; and a DNA segment which corresponds to a segment of the adenoviral genome. The vector also may contain a tripartite leader sequence. The DNA segment corresponding to the adenoviral genome serves as a substrate for homologous recombination with a modified or mutated adenovirus, and such sequence may encompass, for example, a segment of the adenovirus 5 genome no longer than from base 3329 to base 6246 of the genome. The plasmid may also include a selectable marker and an origin of replication. The origin of replication may be a bacterial origin of replication. Representative examples of such shuttle plasmids include pAVS6, shown in Figure 10. A desired DNA sequence encoding a clotting factor may then be inserted into the multiple cloning site to produce a plasmid vector.

This construct is then used to produce an adenoviral vector. Homologous recombination is effected with a modified or mutated adenovirus in which at least the majority of the E1 and E3 adenoviral DNA sequences have been deleted. Such homologous recombination may be effected through co-transfection of the plasmid vector and the modified adenovirus into a helper cell

line, such as 293 cells, by $CaPO_4$ precipitation. Upon such homologous recombination, a recombinant adenoviral vector is formed that includes DNA sequences derived from the shuttle plasmid between the Not I site and the homologous recombination fragment, and DNA derived from the E1 and E3 deleted adenovirus between the homologous recombination fragment and the 3' ITR.

In one embodiment, the homologous recombination fragment overlaps with nucleotides 3329 to 6246 of the adenovirus 5 (ATCC VR-5) genome.

Through such homologous recombination, a vector is formed which includes an adenoviral 5' ITR, an adenoviral encapsidation signal; an E1a enhancer sequence; a promoter; at least one DNA sequence encoding a clotting factor; a poly A signal; adenoviral DNA free of at least the majority of the E1 and E3 adenoviral DNA sequences; and an adenoviral 3' ITR. The vector also may include a tripartite leader sequence. This vector may then be transfected into a helper cell line, such as the 293 helper cell line, which will include the E1a and E1b DNA sequences, which are necessary for viral replication, and to generate infectious adenoviral particles.

In one embodiment, the adenoviral vector comprises an adenoviral 5' ITR; an adenoviral 3' ITR; an adenoviral encapsidation signal; at least one DNA sequence encoding a clotting factor; and a promoter controlling the at least one DNA sequence encoding a clotting factor. The vector is free of the adenoviral E1, E2, E3, and E4 DNA sequences, and the vector is free of DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter; i.e., the vector is free of DNA encoding adenoviral structural proteins.

Such vectors may be constructed by removing the adenoviral 5' ITR, the adenoviral 3' ITR, and the adenoviral encapsidation signal, from an adenoviral genome by standard techniques. Such components, as well as a promoter (which may be an adenoviral promoter or a non-adenoviral promoter), tripartite leader sequence, poly A signal, and selectable marker, may, by standard

techniques, be ligated into a base plasmid or "starter" plasmid such as, for example, pBluescript II KS-(Stratagene), to form an appropriate cloning vector. The cloning vector may include a multiple cloning site to facilitate the insertion of the at least one DNA sequence encoding a clotting factor into the cloning vector. In general, the multiple cloning site includes "rare" restriction enzyme sites; i.e., sites which are found in eukaryotic genes at a frequency of from about one in every 10,000 to about one in every 100,000 base pairs. An appropriate vector in accordance with the present invention is thus formed by cutting the cloning vector by standard techniques at appropriate restriction sites in the multiple cloning site, and then ligating the DNA sequence encoding a clotting factor into the cloning vector.

The vector is then packaged into infectious, nonreplicating, recombinant adenoviral particles, using a helper adenovirus which provides the necessary encapsidation materials. Preferably the helper virus has a defective encapsidation signal in order that the helper virus will not encapsidate itself. An example of an encapsidation defective helper virus which may be employed is described in Grable, et al., J. Virol., Vol. 66, pgs. 723-731 (1992).

The vector and the encapsidation defective helper virus are transfected into an appropriate cell line for the generation of infectious viral particles. Transfection may take place by electroporation, calcium phosphate precipitation, microinjection, or through proteoliposomes. Examples of appropriate cell lines include, but are not limited to, HeLa cells or 293 (embryonic kidney epithelial) cells (ATCC No. CRL 1573). The infectious viral particles (i.e., the adenoviral vector) may then be transduced into eukaryotic cells, such as hepatocytes, whereby the at least one DNA sequence encoding a clotting factor is expressed by the eukaryotic cells in a host.

The vector, consisting of infectious, but replication-defective, viral particles, which contain at least one DNA

sequence encoding a clotting factor, is administered in an amount effective to treat hemophilia in a host. In one embodiment, the vector particles may be administered in an amount of from 1 plaque forming unit to about 10^{14} plaque forming units, preferably from about $1x10^{6}$ plaque forming units to about $1x10^{13}$ plaque forming units. The host may be a human or non-human animal host. The preferred non-human animal host is a mammal, most preferably a dog or a non-human primate.

Preferably, the infectious vector particles are administered systemically, such as, for example, by intravenous administration (such as, for example, via peripheral vein injection) or administered via the portal vein, to the bile duct, intramuscularly, intraperitoneally, or intranasally.

The vector particles may be administered in combination with a pharmaceutically acceptable carrier suitable for administration to a patient. The carrier may be a liquid carrier (for example, a saline solution), or a solid carrier, such as, for example, mirocarrier beads.

As hereinabove stated, the inventors have found that the incorporation of genomic elements into the adenoviral vector provides for enhanced expression of the DNA sequence encoding a clotting factor. Thus, in accordance with another aspect of the present invention, there is provided an adenoviral vector including at least one DNA sequence encoding a heterologous protein, and at least one genomic element affecting the expression of such DNA sequence. The term "genomic element" is used as previously defined. Such genomic elements include, but are not limited to, introns, the 5' untranslated region, and the 3' untranslated region, and portions of said introns and 3' and 5' untranslated regions. The adenoviral vector may be as hereinabove described.

The DNA sequence encoding a heterologous protein may be a DNA sequence which encodes at least one therapeutic agent. The term "therapeutic" is used in a generic sense and includes treating agents, prophylactic agents, and replacement agents.

DNA sequences encoding therapeutic agents which may be placed into the adenoviral vector include, but are not limited to, DNA encoding Factor VIII and Factor IX as hereinabove described; DNA encoding cytokines; DNA sequences encoding tumor necrosis factor (TNF) genes, such as TNF-α; genes encoding interferons such as Interferon-α, Interferon-ß, and Interferon-γ; genes encoding interleukins such as IL-1, IL-1ß, and Interleukins 2 through 14; genes encoding GM-CSF; genes encoding adenosine deaminase, or ADA; genes which encode cellular growth factors, such as lymphokines, which are growth factors for lymphocytes; genes encoding soluble CD4; T-cell receptors; the LDL receptor, ApoE, ApoC, ApoAI and other genes involved in cholesterol transport and metabolism; the alpha-1 antitrypsin (α1AT) gene, the ornithine transcarbamylase (OTC) gene, the CFTR gene, the insulin gene, viral thymidine kinase genes, such as the Herpes Simplex Virus thymidine kinase gene, the cytomegalovirus virus thymidine kinase gene, and the varicella-zoster virus thymidine kinase gene; Fc receptors for antigen-binding domains of antibodies, and antisense sequences which inihibit viral replication, such as antisense sequences which inhibit replication of hepatitis B or hepatitis non-A non-B virus.

Promoters which control the DNA sequence may be selected from those hereinabove described.

In one embodiment, the genomic element and the DNA sequence encoding a heterologous protein are part of the same endogenous gene. For example, the adenoviral vector may include DNA encoding Factor IX and a Factor IX genomic element(s). In another embodiment, the DNA sequence encoding a heterologous protein and the genomic element are taken from different endogenous genes. For example, the adenoviral vector may include DNA encoding Factor VIII and Factor IX genomic elements.

In yet another embodiment, an adenoviral vector may be constructed wherein the adenoviral vector includes DNA encoding a heterologous protein and at least one genomic element(s) from the same endogenous gene. The DNA encoding a heterologous protein

may be modified such that at least one exon is normally present in the DNA encoding the heterologous protein is removed and replaced with one or more exons present in another gene.

Although the scope of this aspect of the present invention is not to be limited to any theoretical reasoning, Applicants believe that, by the inclusion of at least one genomic element in an adenoviral vector including at least one DNA sequence encoding a heterologous protein, one is able to approximate endogenous transcription, RNA processing, and translation of the DNA sequence encoding a heterologous protein, thereby providing for increased expression of the heterologous protein.

The invention will now be described with respect to the following examples; however, the scope of the present invention is not to be limited thereby.

Example 1

Construction of an Adenoviral Vector Including a Factor IX Gene

A. Construction of pG1H9

Plasmid pG1 (described in PCT application No. WO91/10728, published July 25, 1991) (Figure 3) was constructed from pLNSX, Miller, et al., Biotechniques, 7:980-990 (1989). The construction strategy for plasmid pG1 is shown in Figure 1. The 1.6 kb EcoRI fragment, containing the 5' Moloney Sarcoma Virus (MoMuSV) LTR, and the 3.0 kb EcoRI/ClaI fragment, containing the 3' LTR, the bacterial origin of replication and the ampicillin resistance gene, were isolated separately. A linker containing seven unique cloning sites was then used to close the EcoRI/ClaI fragment on itself, thus generating the plasmid pGO. The plasmid pGO was used to generate the vector plasmid pG1 by the insertion of the 1.6 kb EcoRI fragment containing the 5' LTR into the unique EcoRI site of pGO. Thus, pG1 consists of a retroviral vector backbone composed of a 5' portion derived from MoMuSV, a short portion of gag in which the authentic ATG start codon has been mutated to TAG (Bender et al., J. Virol., Vol. 61, pgs. 1639-1649 (1987)), a 54 base pair multiple cloning site (MCS) containing from 5' to 3' the sites EcoRI, NotI, SnaBI, SalI, BamHI, XhoI, HindIII, ApaI, and ClaI, and a 3' portion of MoMuLV from base pairs 7764 to 7813 numbered as described in (Van Beveren et al., Cold Spring Harbor, Vol. 2, pg. 567, 1985). (Figure 2). The MCS was designed to generate a maximum number of unique insertion sites, based on a screen of non-cutting restriction enzymes of the pG1 plasmid, the neo^R gene, the β-galactosidase gene, and $hygromycin^R$ gene, and the SV40 promoter.

pG1 (Figure 3) was cut with BamHI and HindIII. pLIXSN (Palmer, et al, Blood, Vol. 73, No. 2, pgs. 438-445 (February 1989)), which contains a Factor IX gene, an SV40 promoter, and a neo^Rgene, was also cut with BamHI and HindIII. The resulting

BamHI-HindIII fragment, which contains the Factor IX gene, was then ligated to the BamHI-HindIII digested pG1 to form pG1H9. (Figure 4). The Factor IX gene could also have been obtained according to the procedures disclosed in U.S. Patent No. 4,994,371.

B. Construction of pG1H9B

pG1H9B (Figure 6) was constructed so that the 5' portion of the human Factor IX cDNA starting at the first ATG is identical to the natural 5' human Factor IX sequence. Such is not the case for pG1H9 because of an inversion in the DNA sequence.

pG1H9B was constructed as follows. First, a cDNA clone of human Factor IX was generated by PCR amplification of human liver cDNA followed by subcloning into the plasmid pBluescript SK- (Stratagéne, La Jolla, California). The resulting plasmid was designated phfacIX (Figure 5). The 5' end of the Factor IX sequence in this plasmid was then used to replace the 5' end of the Factor IX sequence in G1H9. phfacIX then was cut with BamHI and DraI, and the 334 bp fragment corresponding to the 5' end of the Factor IX cDNA was isolated. pG1H9 was cut with DraI and ClaI and the 1253 bp fragment encoding the 3' end of the Factor IX cDNA was isolated. The two isolated DNA fragments encoding Factor IX cDNA were ligated into the pG1H9 backbone which had been cut with BamHI and ClaI to generate pG1H9B (Figure 6).

C. Construction of pAVS6

The adenoviral construction shuttle plasmid pAvS6 was constructed in several steps using standard cloning techniques including polymerase chain reaction based cloning techniques. First, the 2913 bp BglII, HindIII fragment was removed from Ad-dl327 and inserted as a blunt fragment into the XhoI site of pBluescript II KS-(Stratagene, La Jolla, CA) (Figure 7).

Ad-dl327 is identical to adenovirus 5 except that an XbaI fragment including bases 28591 to 30474 (or map units 78.5 to 84.7) of the Adenovirus 5 genome, and which is located in the E3 region, has been deleted. The E3 deletion in Ad-dl327 is similar to the E3 deletion in Ad-dl324, which is described in Thimmapaya, et al., Cell, Vol. 31, pg. 543 (1983). The complete Adenovirus 5 genome is registered as Genbank accession #M73260, incorporated herein by reference, and the virus is available from the American Type Culture Collection, Rockville, Maryland, U.S.A. under accession number VR-5.

Ad-dl327 was constructed by routine methods from Adenovirus 5 (Ad5). The method is outlined briefly as follows and previously described by Jones and Shenk, Cell 13:181-188, (1978). Ad5 DNA is isolated by proteolytic digestion of the virion and partially cleaved with Xba 1 restriction endonuclease. The Xba 1 fragments are then reassembled by ligation as a mixture of fragments. This results in some ligated genomes with a sequence similar to Ad5, except excluding sequences 28591 bp to 30474 bp. This DNA is then transfected into suitable cells (e.g. KB cells, HeLa cells, 293 cells) and overlaid with soft agar to allow plaque formation. Individual plaques are then isolated, amplified, and screened for the absence of the 1878 bp E3 region Xba 1 fragment.

The orientation of this fragment was such that the BglII site was nearest the T7 RNA polymerase site of pBluescrpt II KS- and the HindIII site was nearest the T3 RNA polymerase site of pBluescript II KS-. This plasmid was designated pHR. (Figure 7).

Second, the ITR, encapsidation signal, Rous Sarcoma Virus promoter, the adenoviral tripartite leader (TPL) sequence and linking sequences were assembled as a block using PCR amplification (Figure 8). The ITR and encapsidation signal (sequences 1-392 of Ad-dl327 [identical to sequences from Ad5, Genbank accession #M73260] incorporated herein by reference) were amplified (amplification 1) together from Ad-dl327 using primers

containing NotI or AscI restriction sites. The Rous Sarcoma Virus LTR promoter was amplified (amplification 2) from the plasmid pRC/RSV (sequences 209 to 605; Invitrogen, San Diego, CA) using primers containing an AscI site and an SfiI site. DNA products from amplifications 1 and 2 were joined using the "overlap" PCR method (amplification 3) (Horton, et al., BioTechniques, 8:528-535 (1990)) with only the NotI primer and the SfiI primer. Complementarity between the AscI containing end of each initial DNA amplification product from reactions 1 and 2 allowed joining of these two pieces during amplification. Next the TPL was amplified (amplification 4) (sequences 6049 to 9730 of Ad-dl327 [identical to similar sequences from Ad5, Genbank accession #M73260]) from cDNA made from mRNA isolated from 293 cells (ATCC Accession No. CRL 1573) infected for 16 hrs. with Ad-dl327 using primers containing SfiI and XbaI sites respectively. DNA fragments from amplification reactions 3 and 4 were then joined using PCR (amplification 5) with the NotI and XbaI primers, thus creating the complete gene block.

Third, the ITR-encapsidation signal-TPL fragment was then purified, cleaved with NotI and XbaI and inserted into the NotI, XbaI cleaved pHR plasmid. This plasmid was designated pAvS6A and the orientation was such that the NotI site of the fragment was next to the T7 RNA polymerase site (Figure 9).

Fourth, the SV40 early polyA signal was removed from SV40 DNA as an HpaI-BamHI fragment, treated with T4 DNA polymerase and inserted into the SalI site of the plasmid pAvS6A-(Figure 9) to create pAvS6 (Figures 9 and 10).

D. Construction of Av1H9B

Factor IX cDNA (Figure 11), which contains the entire protein coding sequence, 26 base pairs of 5' untranslated DNA (assuming translation starts at the third ATG of the message) and 160 base pairs of 3' untranslated DNA, was excised from pG1H9B by restriction digestion with ClaI, followed by filling in the 5'

overhang using Klenow, followed by restriction digestion with SmaI. The Factor IX cDNA could also have been obtained according to the procedures disclosed in U.S. Patent No. 4,994,371.

The fragment encoding Factor IX was isolated by electrophoresis in a 1.0% agarose gel followed by electroelution of the DNA. This fragment was subcloned into pAvS6 which had been linearized with EcoRV and treated with calf intestinal phosphatase. The resulting shuttle plasmid pAvS6H9B (Figure 12), contains the 5' inverted terminal repeat of adenovirus type 5 (Ad 5), the origin of replication of Ad 5, the Ad 5 encapsidation signal, the E1a enhancer, the RSV promoter, the tripartite leader sequence of Ad 5, Factor IX cDNA, the SV40 early polyadenylation signal, and Ad 5 sequences from nucleotide positions 3329-6246.

The recombinant adenoviral vector Av1H9B was generated as depicted in Figure 13. 1.5 x 10^6 293 cells were cotransfected in a 60 mm tissue culture dish with 4μg of the large Cla I fragment of Ad-dl327 (an E3 deletion mutant of Ad 5) and 5μg of shuttle plasmid pAvS6H9B digested with Not I and Kpn I. Transfections were done using BRL's Transfinity calcium phosphate transfection system. Approximately 15 hours after transfection, medium containing DNA/calcium phosphate precipitate was removed from the dishes, the cells were gently washed with PBS, then overlaid with a 1:1 mixture of 2x MEM (GIBCO's 2x Modified Eagle Medium supplemented with 15% FBS) and 2% SeaPlaque agarose.

Plaques were picked using sterile Pasteur pipettes and transferred into 0.1 ml of infection medium (Improved Minimum Essential Medium (IMEM), 1% FBS) in an Eppendorf tube. Resuspended plaques were subjected to three freeze/thaw cycles, then cleared of cell debris by a 15 second centrifugation at full speed in a microfuge.

Recombinant adenovirus was amplified in 293 cells as follows. Approximately 5 x 10^5 293 cells per dish were seeded into 30 mm dishes. The next day medium was removed from the cells and replaced with 0.2 ml of infection medium and 0.1 ml of a resuspended plaque. The plates were incubated with gentle

rocking for 90 minutes in a 37°C, 5% CO_2 incubator. Subsequently, 2 ml of complete medium (IMEM, 10% FBS) were added. Approximately 40 hours later a cytopathic effect was clearly visible; cells were rounded-up and beginning to detach from the plate. Cells and medium were transferred to plastic tubes, subjected to four freeze/thaw cycles, and centrifuged at 2000 x g for 5 minutes. The resulting supernatant is referred to as the crude viral lysate (CVL1).

Viral DNA was isolated from an aliquot of each CVL1, then analyzed for the presence of Factor IX cDNA by PCR, as follows. A 60 µl aliquot of supernatant was transferred into an Eppendorf tube and incubated at 80°C for 5 minutes. The sample was centrifuged at full speed for 5 minutes in a microfuge, then 5 µl of the supernatant were used for PCR analysis. PCR analysis was done using the Perkin Elmer Cetus GeneAmp kit. Two different pairs of primers which amplify different portions of the human Factor IX cDNA were used. All samples yielded the expected amplified band.

Example 2

In vitro and in vivo function of the vector of Example 1

Recombinant adenovirus vectors containing Factor IX cDNA were tested for their ability to express human Factor IX in 293 cells. Approximately 5 x 10^5 293 cells were seeded per 60 mm dish. The next day, medium was replaced with 0.1 ml of recombinant adenovirus and 0.1 ml of infection medium. Plates were incubated for 1 hour with gentle rocking at 37°C in 5% CO_2, followed by addition of 4 ml of complete medium. The cells were gently washed five times with PBS, then 4 ml of complete medium were added. Media samples were collected 24 hours later and centrifuged at 1500 x g for 5 minutes. Supernatants were assayed for human Factor IX by ELISA (Asserachrom IX:Ag ELISA kit, American Bioproducts), and the levels were 445 and 538 ng/ml for

the two samples, demonstrating that the recombinant adenoviral vectors are able to express human Factor IX. Uninfected 293 cells yielded background levels of Factor IX.

One recombinant adenovirus was selected for a large scale virus preparation. Approximately 5 x 10^6 293 cells were seeded onto a 15 cm tissue culture dish. The next day, the medium was replaced with 4 ml of infection medium plus 1 ml of the crude viral stock. Then the plates were incubated at 37°C, 5% CO_2 with gentle rocking for 90 minutes, followed by addition of 15 ml of complete medium. Approximately 40 hours later, when a cytopathic effect was clearly visible, cells and medium were transferred to a 50 ml plastic tube. Cells were lysed by five freeze/thaw cycles and cell debris was removed by centrifugation at 1500 x g for five minutes. This supernatant was termed CVL2.

15 ml of CVL2 then was mixed with 35 ml of infection medium and 5 ml of this mixture was added to each of ten 15 cm plates of nearly confluent 293 cells. The plates were incubated at 37°C, 5% CO_2 with gentle rocking for 1 hour, followed by addition of 15 ml complete medium to each plate. Twenty-four hours later a cytopathic effect was observed; cells were rounded-up, but not lysed. Cells and medium were centrifuged at 2000 x g for 10 minutes. The cell pellet was resuspended in 6 ml of complete medium. Cells were lysed by five freeze/thaw cycles, followed by centrifugation in a SW40 rotor at 7000 rpm for 10 minutes at 4°C. Virus in the supernatant was purified on a CsCl step gradient as follows. 3.0 ml of 1.25 g/ml CsCl in TD buffer (25 mM Tris, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, pH7.5) was placed in an ultraclear Beckman #344060 ultracentrifuge tube. This was underlaid with 3.0 ml of 1.40 g/ml CsCl in TD buffer. The CsCl layers were overlaid with 4.5 ml of viral supernatant. Centrifugation was done at 35,000 rpm, 22°C for 1 hour in a SW40 rotor. Two bands were visible, an upper band that consists of empty capsids and a lower band consisting of intact recombinant adenovirus.

The lower band was collected with a 3 ml syringe and a 21 gauge needle, and then rebanded as follows. 9.0 ml of 1.33 g/ml CsCl in TD buffer was placed into an ultracentrifuge tube. This was overlaid with the virus collected from the first spin. Centrifugation was done at 35,000 rpm, 22°C for 18 hours. The opalescent band was collected as above and glycerol was added to a final concentration of 10%. The adenovirus was dialyzed against one liter of 10 mM Tris pH 7.4, 10 mM $MgCl_2$, and 10% glycerol at 4°C. Dialysis was done for 4 hours and the buffer was changed three times at one hour intervals. The virus was recovered and stored at -70°C in aliquots in sterile Eppendorf tubes. The titer of this virus preparation was $9.6x10^9$ pfu/ml.

In the first in vivo experiment, the recombinant adenovirus Av1H9B was injected into three C57BL/6 mice by three different methods: an intraparenchymal injection into the liver, infusion into the portal vein, and infusion into the tail vein.

The animal which received an intraparenchymal injection was anesthetized under Metofane. A longitudinal incision approximately 7 mm in length was made just below the xiphoid. Pressure was applied to the flanks causing protrusion of the median and left lateral lobes. For injection, 0.1 ml of virus ($1x10^9$ pfu) was diluted to 1.0 ml with Hanks Balanced Salt Solution (HBSS). The virus was injected into 4 different sites of the liver: 0.25 ml was injected into each half of the median lobe and into the left and right sides of the left lateral lobe. Each injection was done over approximately one minute. Upon removal of the needle, hemostasis was achieved by placing small pieces of gelfoam over the injection site. After 2 minutes, the gelfoam was removed, the liver was gently placed into the abdominal cavity, and the skin incision was closed with autoclips. Animals awakened within several minutes of surgery and were ambulatory within one hour.

The animal which received a portal vein infusion of AdH9B was anesthetized under Metofane. A midline longitudinal incision was made from the xiphoid to just above the pelvis. The

intestines were gently externalized to the left side of the animal with wet cotton tip applicators. An 0.1 ml aliquot of virus ($1x10^9$ pfu) was diluted to 1.0 ml with HBSS. The viral suspension was infused over 30 seconds into the portal vein using a 1 ml syringe and a 27 gauge needle. A 3 x 3 mm piece of gelfoam was placed over the injection site. The needle then was withdrawn. Hemostasis was achieved by applying mild pressure to the gelfoam for 5 minutes using a wet cotton tip applicator. The gelfoam was left in place. The intestines were gently returned to the abdominal cavity. The incision was closed using autoclips. The animal awakened within 30 minutes of surgery and was ambulatory within 1 hour.

A tail vein infusion of Av1H9B was performed using 0.1 ml of virus ($1x10^9$ pfu) diluted to 1.0 ml with HBSS. The viral suspension was infused over a ten second period using a 27 gauge needle.

The animals which received an intraparenchymal injection and portal vein infusion, as well as a control mouse which received no virus, were bled via the retro-orbital plexus on days 2, 6, and 9 after virus delivery. The animal which received a tail vein infusion was bled 2 days after virus delivery. Plasma levels of human Factor IX were determined by ELISA. The results are shown in Figure 14.

At this point, it was important to determine vector levels in the livers of the mice. Therefore, the animals which received an intraparenchymal injection and a portal vein infusion and the negative control mouse were sacrificed on day 9 after infusion and the mouse which received a tail vein injection was sacrificed on day 2 after infusion. The liver of each mouse was removed and extensively minced with a razor blade. One-half of each liver was placed into a 15 ml conical tube and 1.0 ml of lysis buffer (10 mM Tris, 0.14 M NaCl, pH 8.6) was added. The tissue was homogenized using a 1 ml syringe and a 20 gauge needle. Next, 1.0 ml of 2x PK buffer (200 mM Tris pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% (w/v) SDS, and 500 μg/ml proteinase K) was added. The

tube was inverted several times, then incubated at 37°C overnight. The samples were extracted twice with phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1). DNA was ethanol precipitated, washed with 70% ethanol, and resuspended in 10 mM Tris, pH 7.5, 1 mM EDTA.

A Southern analysis was performed to quantitate the levels of vector in the liver. Ten micrograms of each DNA sample were cut with BamHI. The digested DNA samples were subjected to electrophoresis in an 0.8% Seakem agarose gel in 40 mM Tris, 20 mM NaAcetate, 1 mM EDTA, pH7.5.

After electrophoresis, the gel was treated with 0.2 N NaOH, 0.6 M NaCl for 1 hour, then neutralized with 1 M Tris pH 7.4, 0.6 M NaCl for 30 minutes. The DNA was transferred to a nylon membrane by blotting in 10 x SSC. The nylon membrane was baked at 80°C for 1 hour in a vacuum oven. It was prehybridized for 3 hours at 42°C in 5x Denhardt's, 5x SSC, 50 mM NaPhosphate pH 6.5, 250 μg/ml salmon sperm DNA, 0.1% SDS, and 50% formamide. The membrane was hybridized for 24 hours at 42°C in 1x Denhardt's, 5x SSC, 20 mM NaPhosphate pH 6.5, 100μg/ml salmon sperm DNA, 0.1% SDS, 50% formamide, and 33 μCi of random primer labeled human Factor IX cDNA. Random primer labeling was performed using the BRL kit. The membrane was washed in 2x SSC, 0.1% SDS for 20 minutes at room temperature, followed by a 30 minute wash in 2x SSC, 0.1% SDS at 50°C, and then a 30 minute wash in 0.1x SSC, 0.1% SDS at 68°C. The membrane was exposed to film for 16 hours, then developed. A copy of the autoradiogram is shown in Figure 15. All three routes of administration yielded the same results. The Factor IX bands were the appropriate size with an intensity that indicated an average of 5-10 copies per liver cell.

Example 3

In vivo expression of Factor IX in mice injected with Av1H9B

A second large scale virus preparation of Av1H9B was performed using the same protocol described above, except that 28 15 cm plates of 293 cells were used to amplify the virus. This preparation yielded a much thicker opalescent band upon CsCl gradient centrifugation than the first virus preparation. The titer of this virus preparation was 1.1×10^{11} pfu/ml.

A second in vivo experiment, designed to follow the time course of expression, was initiated using the new Av1H9B preparation. Virus was administered to mice as described above, except that 0.1 ml of a virus suspension (1×10^{10} pfu) was diluted to 1.0 ml with infection medium. Twenty-seven mice received an injection of recombinant adenovirus: 20 mice received a tail vein injection, 18 with Av1H9B and 2 with Av1lacZ4 (encoding ß-galactosidase), 4 mice received an intraparenchymal injection of the liver, and 3 received an intramuscular injection. A negative control mouse was not injected. The animals were bled once a week for seven weeks. Plasma levels of human Factor IX are shown in Table I.

As shown in Table I, IP means intraparenchymal injection of Av1H9B, TV means a tail vein injection of Av1H9B, IM means an intramuscular injection of Av1H9B, LacZ means a tail vein injection of Av1lacZ4, and NI means no injection (control).

Table I

ng/ml Factor IX in plasma

Mouse	Injection	Week 1	Week 2	Week 3	Week 4
1.	TV	376	475	281	171
2.	TV	270	500	392	336
3.	TV	229	374	---	---
4.	TV	240	---	---	---
5.	TV	362	---	---	---
6.	TV	346	---	---	---

Table I (continued)

ng/ml Factor IX in plasma

7.	TV	303	422	252	142
8.	TV	260	573	394	220
9.	TV	353	376	273	149
10.	TV	321	357	270	246
11.	TV	431	482	233	203
12.	TV	347	332	---	---
13.	TV	135	244	126	61
14.	TV	261	294	187	148
15.	TV	212	269	132	91
16.	TV	207	255	214	176
17.	TV	278	218	151	149
18.	TV	170	308	---	---
19.	IM	0.9	3.0	0.0	0.0
20.	IM	1.0	2.7	0.0	0.0
21.	IM	1.1	2.6	1.0	1.2
22.	IP	364	316	174	131
23.	IP	211	308	134	66
24.	IP	305	252	155	206
25.	IP	527	406	133	94
26.	LacZ	0.0	2.8	1.5	0.4
27.	LacZ	0.0	2.6	2.0	1.2
28.	NI	0.0	2.6	1.5	0.5

Mouse	Injection	Week 5	Week 6	Week 7
1.	TV	98	34	16
2.	TV	187	67	16
3.	TV	---	---	---
4.	TV	---	---	---
5.	TV	---	---	---
6.	TV	---	---	---
7.	TV	---	---	---
8.	TV	197	60	25
9.	TV	131	90	46
10.	TV	179	76	16
11.	TV	---	---	---

12.	TV	---	---	---
13.	TV	84	62	17
14.	TV	133	65	26
15.	TV	94	64	33
16.	TV	---	---	---
17.	TV	92	57	23
18.	TV	---	---	---
19.	IM	0.0	2.0	0.0
20.	IM	0.0	2.0	4.1
21.	IM	0.1	2.0	0.0
22.	IP	112	53	48
23.	IP	42	28	19
24.	IP	299	203	154
25.	IP	57	21	13
26.	LacZ	0.0	1.5	5.0
27.	LacZ	0.0	1.5	4.0
28.	NI	1.4	1.7	0.5

Example 4

Assay for biological activity of human Factor IX

The biological activity of human Factor IX in mouse plasma was determined by using an immunocapture, chromogenic assay. A 96-well microtiter plate was coated with a BGIX1 monoclonal antibody obtained from Elcatech, Inc., which recognizes, but does not inactivate, human Factor IX. Coating was done by adding 100μl of a 10μg/ml suspension of the antibody to each well and incubating at room temperature overnight. Plasma samples obtained from Mouse 7 in Example 3 two weeks after injection with the recombinant adenovirus (100μl of 1:5 and 1:10 dilutions) were added to the wells and human Factor IX was allowed to bind. The wells were washed to remove unbound material, and captured human Factor IX was activated by adding 100μl of a 2μg/ml suspension of Factor XIa (Enzyme Research Labs) and incubating at 37°C for 30 minutes. The wells were washed, and then 100μl of a mixture containing 5.0μg phospholipid (Kabi Pharmacia, Franklin, Ohio), 0.1 unit Factor X (Kabi), 0.5 unit Factor VIII (Elcatech), 3.4μg I-2581 thrombin inhibitor (Kabi) and 2.5mM $CaCl_2$ were added. The plate was incubated at 37°C for 30 minutes, during which time Factor X was converted to Factor Xa. 100μl of 0.5mM N-N-alpha-benzyloxycarbonyl-D- arginyl-L-glycyl-L-arginine-p-nitroanilide-dihydro choloride, a chromogenic Factor Xa substrate, then was added and the plate was incubated at room temperature for ten minutes. The color development was stopped by adding 50μl of 50% acetic acid. The absorbance at 405nm was determined using a Bio-Rad microplate reader. Standard curves (log-log and linear-linear) were generated using normal pooled human plasma, assuming Factor IX levels of 5000ng/ml. Biologically active Factor IX was determined to be 511ng/ml according to the log-log method, and 415ng/ml according to the linear-linear method. Such results are within experimental error, and indicate that essentially all of

the total Factor IX antigen determined in Example 3 (422ng/ml) is biologically active.

Example 5

Construction of Adenoviral Vector Including DNA Encoding a Factor VIII Derivative

pAVS6H81 was constructed from pMT2LA (Figure 16) and pAVS6. (Figure 10). pMT2LA (Genetics Institute, Cambridge, Mass.) includes cDNA encoding a derivative of human Factor VIII in which the B domain of Factor VIII is deleted. Such cDNA is further described in Toole, et al., Nature, Vol. 312, pgs. 342-349 (November 1984), Vehar, et al., Nature, Vol. 312, pgs. 337-342 (November 1984), and Toole, et al., PNAS, Vol. 83, pgs. 5939-5942 (August 1986). The cDNA is controlled by a Rous Sarcoma Virus promoter. The 4.6 kb cDNA (Figure 17) contains no natural 5' untranslated DNA, and 216 bp of 3' untranslated DNA. The B domain deletion removes nucleotides 2334-4973 of the coding sequence of the full length Factor VIII. The cDNA for B domain deleted Factor VIII could also have been obtained according to the procedures disclosed in U.S. Patent No. 4,868,112.

The cDNA was excised from the plasmid pMT2LA by restriction digestion with XhoI and SalI. The ends were filled in using Klenow, and the fragment encoding the Factor VIII derivative was isolated on an 0.8% agarose gel, followed by electroelution. This fragment was subcloned into the EcoRV site of pAVS6 (Figure 10) to generate pAVS6H81. (Figure 18.)

The recombinant adenoviral vector Av1H81 is generated as depicted in Figure 19. 1.5 x 10^6 293 cells are cotransfected in a 60 mm tissue culture dish with 4 μg of the large ClaI fragment of Ad dl 327 and 5 μg of pAvS6H81 digested with NotI. Transfections are done using BRL's Transfinity calcium phosphate transfection system. Approximately 15 hours after transfection, medium containing DNA/calcium phosphate precipitate is removed

from the dishes, the cells are gently washed with PBS, then overlaid with a mixture of 2x MEM and 2% Sea Plaque agarose.

Recombinant adenovirus can be prepared from plaques and analyzed by PCR for the presence of human Factor VIII cDNA.

Example 6

Generation of Adenoviral Vectors Including DNA Encoding Factor VIII Plus Genomic Elements

A. Construction of pAvALH81

The mouse albumin promoter (Zaret, et al., Proc. Nat. Acad. Sci. USA, Vol. 85, pgs. 9076-9080 (1988)), containing 3.5 copies of a liver-specific transcription factor binding site (eG binding sites, Liu et. al., Mol. Cell. Biol., Vol. 11, pgs. 773-784 (1991) and Di Persio, et al., Mol. Cell. Biol., Vol. 11, pgs. 4405-4414 (1991)) was PCR amplified from pAT2-3eG (Figure 20, provided by Kenneth Zaret, Brown University, Providence, Rhode Island) using oligo MGM8.293,

5'-GGC TAG ACG CGT GCT ATG ACC ATG ATT ACG AA-3'

complementary to nts 4281-4299 of pAT2-3eG with the addition of an MluI restriction site, as the 5' oligo, and oligo MGM5.293,

5'-GGT ACG GAT CCA TCG ATG TCG ACG CCG GAA AGG TGA TCT GTGT-3'

complementary to nts 5231-5212 of pAT2-3eG with the addition of BamHI, ClaI, and SalI restriction sites, as the 3' oligo. The PCR product was cut with MluI and BamHI and inserted into pAVS6 (Figure 10) cut with MluI and BamHI to generate pAVAL1 (Figure 21). The sequence of the 964 bp PCR-generated albumin promoter has been verified by sequencing. In addition, at least 50 bp on either side of the MluI site (nt 428) and BamHI site (nt 1392) in

pAVAL1 (Figure 21) have also been verified by sequencing. The plasmid pAT-2-3eG is prepared according to the procedures disclosed in DiPersio, et al., Mol. Cell. Biol., 11:4405-4414 (1991) and Zaret, et al., Proc. Nat. Acad. Sci., Vol. 85, pgs. 9076-9080 (1988), which disclose the preparation of a mouse albumin promoter with two copies of a liver-specific transcription factor binding site. The plasmid pAT2-3eG has been deposited under the Budapest Treaty in the American Type Culture Collection, 1230 Parklawn Drive, Rockville, Maryland 20892, and assigned accession number 69603.

The ITR, encapsidation signal (see construction of pAVS6) and the albumin promoter were removed from pAVAL1 by digestion with NotI (the ends were filled in with T4 DNA polymerase) and SalI, and inserted into pGEM(sac) (Figure 22), cut with SalI and SmaI to generate pGEMalb (Figure 24) (pGEM(sac) was created by cutting pGEM (Figure 23, Promega) with SacI, and blunting the ends with T4 DNA polymerase and religation, thereby removing the SacI site.) A 1914 bp fragment, containing the 5' region of the B-domain deleted factor VIII cDNA was isolated from pMT2LA (Figure 16) by digestion with BamHI (filling in the 5' end with T4 DNA polymerase) and digestion with XhoI, and inserted into pGEMalb digested with HindIII (filled in with T4 DNA polymerase) and SalI, to generate pGEMalbF8B (Figure 25). pGEMalbF8B was cut with MluI and SpeI, and the resulting 1556 bp fragment was inserted into pAvS6H81 (Figure 18), cut with MluI and SpeI, to generate the adenovirus shuttle plasmid, pAvALH81 (Figure 26). At least 50 bp on either side of the MluI site (nt 429) and SpeI site (nt 1985) have been verified by sequencing of Av1ALH81 viral DNA (see below). The sequence of the Factor VIII B-domain deleted cDNA has been verified by sequencing of bases 1075 to 5732 from the original plasmid, pMT2LA (Figure 16) obtained from Genetics Institute. It should be noted that this sequence differs from the sequence reported by Genetics Institute by two bases. One base change, nt 1317 of pMT2LA was reported by Genetics Institute to be a T (Toole et. al., Nature, Vol. 312,

pgs. 342-347 (1984) and by Wood et. al., Nature, Vol. 312, pgs. 330-337 (1984) to be an A. In addition, nt 5721 of pMT2LA, reported by Genetics Institute to be a T, was deleted, thus creating a BamHI site within the Factor VIII 3' untranslated region. This mutation does not change the Factor VIII coding region.

B. Construction of pAvAPH81

A 1913 bp fragment was isolated from pAVS6H81 (Figure 18) by digestion with Bam HI, and inserted into pGEM(sac) (Figure 22) cut with Bam HI, to create pGemF8B2 (Figure 27). The ApoA1 promoter, first exon (untranslated), first intron, and second exon to the ATG (Genbank #X07496) were PCR amplified using pBGS19-AIgI (Figure 28) as the template. pBGS19-AIgI (Figure 28) was constructed in two steps: 1) The 13 kb SalI fragment was removed from Lambda A1 103 (Swanson, et. al., Transgenic Research, Vol. 1, pgs. 142-147 (1992), and inserted into pUC19 (Figure 29, Gibco BRL) to generate pUC19-AIgI (Figure 30). 2) The 2 kb SmaI fragment was isolated from pUC19-AIgI (Figure 30) and inserted into pBGS19 (Figure 31) to generate pBGS19-AIgI (Figure 28). pBGS19 (ATCC No. 37437) is a kanamycin analog of pUC19. PCR-amplification of pBGS19-AIgI was performed using oligo SSC1.593,

5'GCT CTA GAA CGC GTC GGT ACC CGG GAG ACC TGC AAG CC-3'

complementary to bases 5862 to 16 of pBGS19-AIgI, containing an XbaI and a MluI site, as the 5' oligo, and a 3' oligo SSC2.593,

5'-GGA ATT CGA GCT CTAT TTG CAT CCT GAA GGG CCG TGG GGA CCT GG-3'

complementary to human factor VIII (Genbank #KO1740, nts 151-165 (to the SacI site), and nts 463-487 of pBGS19-AIgI, complementary to the ApoA1 gene (Genbank #XO7496) with the addition of a SacI and an EcoRI site. The PCR fragment was digested with XbaI and SacI and the resulting 509 bp fragment was inserted into pGemF8B2

(Figure 27) digested with XbaI-SacI, to generate pGemAPF8B (Figure 32). pGemAPF8B was then digested with MluI-SpeI, and the resulting 1084 bp fragment was ligated into pAVS6H81 (Figure 18) cut with MluI and SpeI, to generate the shuttle plasmid, pAvAPH81 (Figure 33). The sequence of pAvAPH81, from nts 290 to 1619, which include the PCR-generated ApoA1 promoter region, and all cloning junctions, has been verified.

C. Construction of pAvALAPH81

A SalI site was added upstream from the ApoA1 transcription initiation site by PCR amplification of pGemAPF8B (Figure 32) using a 5' oligo SSC3.593,

5'-GAA TTC GTC GAC AGA GAC TGC GAG AAG GAG GTG CG-3'

complementary to the ApoA1 gene (Genebank #X07496) and nts 252-274 of pBGS19-A1g1 (Figure 28) with the addition of an EcoRI and a SalI site, and a 3' oligo, SSC2.593 (see above). The PCR fragment was digested with SalI-SacI, and the resulting 250 bp fragment was inserted into pGemF8B2 (Figure 27) cut with SalI-SacI, to create pGemAPexF8B (Figure 34). The plasmid, pALAPF8B (Figure 35) was generated by a 3-way ligation of the 953 bp MluI-SalI fragment isolated from pGEMalb (Figure 24), the 825 bp SalI-SpeI fragment isolated from pGemAPexF8B (Figure 34), inserted into pGemAPF8B (Figure 32) cut with MluI-SpeI. The 1778 bp MluI-SpeI fragment was isolated from pALAPF8B (Figure 35) and inserted into pAVS6H81 (Figure 18) to generate the shuttle plasmid, pAvALAPH81 (Figure 36).

D. Generation of recombinant adenovirus vectors

The recombinant adenoviral vector, Av1ALH81, was generated as outlined in Figure 37. 2 x 10^6 293 cells were cotransfected in a 100 mm tissue culture dish with 10 μg of the large ClaI fragment of Ad-dl327, and 10 μg of the undigested shuttle plasmid, pAvALH81 (Figure 26). Transfections were performed

using the Transfinity calcium phosphate transfection system from BRL. Approximately 12 hrs after DNA addition, the cells were washed 2X with 1X PBS, then overlaid with a 1:1 mixture of 2X MEM (GIBCO'S 2X Modified Eagle Medium supplemented with 15% FBS) and 2% SeaPlaque agarose.

Plaques were harvested with sterile Pasteur pipettes and transferred into 0.1 ml of infection medium (Improved Minimum Essential Medium ([IMEM], 2% FBS) in an Eppendorf tube, and subjected to three rounds of freeze/thaw cycles. Cell debris was removed by a 15 second centrifugation at full speed in a microfuge.

Plaques were screened for the presence of recombinant adenovirus as follows. Approximately 5 x 10^5 293 cells were seeded per well of 6-well tissue culture plates. The following day, media was removed from the cells and replaced with 0.4 ml of infection medium and 0.05 ml of the resuspended plaque. The plates were incubated with rocking, for 90 min. in a 37°C/5% CO_2 incubator, after which 2 ml of complete medium (IMEM, 10% FBS) were added. When the cytopathic effect (CPE) was complete, cells were rounded and becoming detached from the plate (approximately 40-120 hrs after infection), cells and medium were transferred to 15 ml conical tubes, and centrifuged at 1000 rpm for 5 min. to pellet cells. The medium was removed from the cell pellet, and the cells were processed as follows.

Cells were resuspended in 250 µl of PK buffer (5mM Tris pH 8.0, 5mM EDTA, pH 8.0, and 0.5% SDS) plus 250 µl of Proteinase K (1mg/ml), and incubated 4 hrs or overnight at 37°C. The solution was transferred to Eppendorf tubes and extracted with an equal volume of phenol 1X, phenol-$CHCl_3$ 1X, and $CHCl_3$ 1X, and ethanol precipitated. Pellets were resuspended in 50 µl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0), and genomic DNA was analyzed by restriction digestion. One plaque yielded the expected product.

This plaque of Av1ALH81 was plaque purified as follows. 5 x 10^5 293 cells per well were plated on a 6-well tissue culture

plate. The next day, medium was removed from the cells, and 0.4 ml of infection medium containing 3 varying amounts of the resuspended plaque were added to each well, 25 μl, 2.5 μl, and 0.25 μl. The plate was rocked for 1.5 hrs in a 37°C/5% CO_2 incubator, after which the media was removed, and the wells were overlaid with a 1:1 mixture of 2X MEM and 2% SeaPlaque agarose as described. Plaques were visible in all wells 9 days after infection. Several plaques were picked from the lowest dilution well (0.25 μl of resuspended plaque), and screened for the presence of AvlALH81 as described. All plaques yielded the expected virus.

One plaque-purified plaque was selected for large scale virus preparation. 5 x 10^5 cells were plated in each well of a 6 well plate and the next day infected with 50 μl of the resuspended plaque-purified plaque as described. Five days after infection, the CPE was complete, cells and medium were transferred to 15 ml conical tubes and subjected to four freeze/thaw cycles, then cleared of cell debris by centrifugation at 1000 rpm for 5 min. The resulting supernatant is referred to as crude viral lysate #1 (CVL-1). This CVL was used to infect a 150 mm plate containing approximately 2 x 10^7 293 cells as follows.

Medium was replaced with 1.25 ml of Infection Medium plus 100 μl of CVL, and the plate was rocked for 1.5 hrs as described, after which 20 mls of complete medium was added. Approximately 20 hrs after infection, the CPE was complete, and cells and medium were transferred to a 50 ml conical tube, spun for 5 min at 1000 rpm, supernatant was removed and saved, and the cell pellet was resuspended in 5 ml of supernatant. After four freeze/thaw cycles, the CVL was removed of cell debris as described. The resulting supernatant is referred to as CVL-2. 30-80% confluent 150 mm plates of 293 cells were infected using the CVL-2 as follows.

600 μl of CVL-2 was added to 38 mls of Infection Medium, medium was removed from the plates, and replaced with 1.25 ml of

the CVL-2-Infection Medium mixture. Plates were rocked for 1.5 hrs as described, after which 20 mls of complete medium was added to each plate. The CPE was complete after 30 hrs and cells were processed as follows. Cells and media were harvested into 250 ml centrifuge bottles and spun at 1500 rpm for 10 min. The cell pellet was resuspended in 20 mls of supernatant. Cells were lysed by five freeze/thaw cycles, followed by centrifugation in a SW40 rotor at 7000 rpm for 10 min at 4°C. Virus in the supernatant was purified on a CsCl step gradient as follows.

3.0 ml of 1.25 g/ml CsCl in TD buffer (25 mM Tris, 137 mM NaCl, 5mM KCl, 0.7 mM Na_2HPO_4 Ph 7.5) was placed in four ultraclear Beckmann #344060 ultracentrifuge tubes. 3.0 ml of 1.4 g/ml CsCl in TD buffer was then underlaid. The CsCl layers were overlaid with 5.0 ml of viral supernatant. Centrifugation was performed at 35,000 rpm, 22°C for 1 hr in a SW40 rotor. Two bands were visible, an upper band consisting of empty capsids, and a lower band composed of intact recombinant adenovirus.

The lower band was collected with a 3 ml syringe and a 18 gauge needle, and then rebanded by placing 8.0 ml of 1.33 g/ml CsCl in TD buffer into two ultracentrifuge tubes, and overlaying with virus collected from the first spin. Centrifugation was performed at 35,000 rpm, 22°C for 18 hrs. The viral band was collected as described and glycerol was added to a final concentration of 10%. The virus was dialyzed against one liter of 10 Mm Tris pH 7.4, 10 Mm $MgCl_2$, and 10% glycerol at 4°C. Dialysis lasted for four hours with buffer changes every hour. The virus was recovered and stored at -70°C in aliquots in sterile Eppendorf tubes. The titer of this virus preparation (Lot # MS1-1) was 1.5×10^{11} pfu/ml. A second Av1ALH81 viral prep was made in a similar manner as described, again using 600 µl of CVL-2 and 30-150 mm plates of 80% confluent 293 cells. The titer of the second prep (Lot # MS1-2) was 9×10^{10} pfu/ml.

At this stage, the viral DNA is checked for deletions or rearrangements. Studies utilizing retroviral vectors containing Factor VIII cDNA sequences have been shown to delete and/or

rearrange portions of the Factor VIII CDNA at high frequencies (Lynch et. al., 1993), and similar rearrangements may be seen with Factor VIII-containing adenoviral vectors. Therefore, viral DNA was isolated from both lots (MS1-1, and MS1-2) of AvALH81 as follows. 100 μl of purified virus was added to 100 μl of TE, 5 μl of 10% SDS, and 20 μl of 10 mg/ml Proteinase K (Sigma), and digested overnight at 37°C. The viral DNA was extracted with an equal volume of phenol 1X, phenol-$CHCl_3$ 1X, and $CHCl_3$ 1X, then the supernatant was put over a Centricon 10 concentrator (Amicon) and the volume was increased to 2 mls with TE, and spun at 5000 rpm for one hour. The centricon was then washed with 2 mls of TE, and spun for 30 min at 5000 rpm. DNA was recovered by inverting the upper chamber of the centricon, inserting into the collection tube, and centrifugation at 3000 rpm for 5 min. Final volume of the purified DNA was increased to 100 μl, and the DNA concentration was calculated. 10 μg of MS1-1, MS1-2, and dl327 DNA was digested overnight with BamHI, HindIII, or, NdeI, and run on a 0.8% agarose gel. DNA fragments were visualized with ethidium bromide staining (Figure 38). Both Av1ALH81 lots look the same, and all restriction fragments are of the predicted sizes. Therefore, unlike the Factor VIII-containing retroviral vectors (Lynch et. al., 1993), the genome of Av1ALH81 is stable.

The recombinant adenoviral vector Av1ALAPH81 was generated as outlined in Figure 39. 2 x 10^6 293 cells were cotransfected in a 100 mm tissue culture dish with 10 μg of the undigested shuttle plasmid, pAvALAPH81 (Figure 36). Generation of adenoviral vector Av1ALAPH81 then was carried out in the same manner as the generation of adenoviral vector Av1ALH81. Av1APH81 can be generated in the same manner.

Example 7

In Vivo Expression of Adenoviral Vectors Including DNA Encoding Factor VIII Plus Genomic Elements

A. Factor VIII Tri-Sandwich ELISA

Before Av1ALH81 could be tested for Factor VIII expression *in vivo*, in mice, or *in vitro*, in tissue culture cells, it was necessary to develop an assay capable of measuring low levels of human Factor VIII present in mouse plasma. The only commercially available Factor VIII assay, Coatest (Kabi Pharmaceuticals) measures the biological activity of Factor VIII protein, and can be used to measure Factor VIII levels in tissue culture cells. However, Coatest cannot distinguish human Factor VIII from animal Factor VIII and, therefore, is not useful for measuring human Factor VIII in animal plasma. To measure the amount of human Factor VIII present in tissue culture medium or animal plasma samples, a quantitative Factor VIII tri-sandwich ELISA was developed. This ELISA can measure human Factor VIII specifically in mouse and dog plasma, and can measure reproducibly Factor VIII concentrations down to 1.0 ng/ml. The assay is performed as follows.

A 96 well microtiter plate is coated with two commercially available monoclonal antibodies with unique epitopes for Factor VIII protein and incubated overnight at 4°C to allow adherence to the plastic wells. 0.5 ug of each antibody (N7, Biodesign; and ESH2, American Diagnostica) were diluted in dilution buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, sterile H_2O to one liter, pH 9.6), and 100 µl of the dilution was added to each well. These antibodies constitute the primary antibody. The use of two capture antibodies, that act cooperatively to increase the sensitivity of the assay, has not been described previously. After the overnight incubation, the plate is washed gently 3X with 200 µl per well of 1X PBS and blotted dry. Blocking agent [1X PBS, 10% Horse Serum (heat inactivated, BioWhittaker), and 1 mm $CaCl_2$] is added, and incubated for two hours at room temperature, after which the plate is washed with 200 µl per well of washing solution [1X PBS, 0.05% Tween 20 (Sigma)] 3X and blotted dry. Samples then are diluted appropriately (usually a 5-fold

dilution) in TNTC (50 mm Tris pH 7.2, 5 mm $CaCl_2$, 0.1% Tween 20, 0.5 M NaCl), aliquoted into each well, and incubated for one hour at 37°C, after which the wells are washed with the washing solution as described. The secondary antibody, which is diluted serum from a hemophiliac (a 1:1000 dilution in the blocking agent solution, 100 µl per well) containing Factor VIII inhibitor antibodies, is added and allowed to bind for one hour at 37°C, after which the wells are washed with the washing solution as described. The third antibody, a commercially available goat anti-human IgG antibody conjugated to horseradish peroxidase (goat anti-human IgG-HRP, Pierce, 0.8 mg/ml, diluted 1:5000 in blocking agent, 100 µl per well), is added, and incubated for one hour at 37°C. The excess antibody then is washed out of the wells (as described, but 5X) and the substrate tetramethylbenzidine (TMB) (Kirkegaard and Perry Labs; 100 µl of the commercially available solution), which when cleaved by the HRP, yields a blue color, is added to each well. The level of color that develops is proportional to the amount of Factor VIII present in the sample. The reaction is stopped, after 2-3 minutes with an acid stop solution (TMB stop solution, Kirkegaard and Perry Labs, 100 µl per well) and the absorbance is determined using a microtiter plate reader. An example of a typical standard curve, using full-length human Factor VIII protein concentrations ranging from 0.078 ng/ml to 40.00 ng/ml is displayed in Figure 40.

B. Half-Life Study of B-Domain Deleted Factor VIII in Mouse Plasma

After development of this extremely sensitive Factor VIII ELISA, a half-life study of B-domain deleted (BDD) Factor VIII in mouse plasma was undertaken. It had been reported (Hoeben et. al., 1993) that the half-life of human Factor VIII in mice was only one hour, compared to the 10 to 12 hour half-life of full-length human Factor VIII in humans and dogs (Brinkhous, et al.,

PNAS, Vol. 82, pgs. 8752-8756 (1985)). The determination of the half-life of BDD human Factor VIII in mice was important for the subsequent evaluation of the efficacy of Av1ALH81 for gene therapy protocols utilizing the mouse as an *in vivo* model.

The half-life study was performed twice. In the first experiment (Figure 41) five C57bl/6 female mice were injected via tail vein with 400 ng of BDD Factor VIII protein. Blood was drawn at 0.5, 1.5, 2.5. and 6.5 hours post injection. In the second experiment (Figure 42), designed to focus on the 2 to 14 hour post injection time range, four C57bl/6 female mice were injected via tail vein, with 500 ng of BDD Factor VIII. Blood was drawn at 2, 5, 8, 12, and 14 hours post injection and plasma analyzed for the presence of human Factor VIII antigen. The results are displayed in Figures 41 and 42. The half-life of human Factor VIII in mice was calculated to be 4-5 hours. This result contrasts with the half-life calculated by Hoeben et. al. (1993). However, in the study by Hoeben et. al. (1993), the half-life of Factor VIII in mice was analyzed over only a 2 hour time period. In the study reported here, it was found that there was a sharp decrease (half-life 1.7 hours) in the level of Factor VIII antigen in mouse plasma between 30 minutes and 2 hours post injection (Figure 41), with the decay leveling off to a half-life of 4-5 hours at subsequent time points (Figures 41 and 42). Therefore, the results indicate that the half-life of human BDD Factor VIII in mice is approximately 2-3 times shorter than the human Factor VIII half-life in humans and dogs.

C. Production of Biologically Active Factor VIII In Vitro

To determine if Av1ALH81 transduction resulted in the production of biologically active Factor VIII *in vitro*, 293 cells were infected with CVL-1, generated from two separate plaques of plaque-purified Av1ALH81 as follows. The medium was removed from 3-150 mm plates of 293 cells containing 1.5 x 10^7 cells, and replaced with 1.15 mls of Infection Medium, plus 100 μl of CVL-1

from either Av1ALH81 plaque (plaque 1 or plaque 2), or, for the negative control plate, 1.25 mls of Infection Medium. Plates were rocked for 1.5 hrs. as described, after which 20 mls of complete medium was added to each plate. 1.0 ml of medium was collected from each plate at 0, 12, and 24 hr. time points, and analyzed for the presence of Factor VIII antigen, using the human Factor VIII-specific ELISA, described above, and analyzed for biological activity, using the Coatest Assay (Kabi Pharmaceuticals). The results are displayed in Table II below.

Table II

Expression of Factor VIII in Av1ALH81 Transduced 293 Cells

		Assay	
Virus	Time (hrs)	ELISA (ng/ml) total antigen	Coatest (ng/ml)* biological activity
Av1ALH81	0	0.0	0.0
plaque 1	12	9.8	6.9
	24	10.2	1.0
Av1ALH81	0	0.0	0.0
plaque 2	12	22.1	7.6
	24	24.3	0.0
No virus	0	0.0	0.0
	12	0.0	0.0
	24	0.0	0.0

* converted from units in which one unit of activity equals 200ng/ml of Factor VIII.

As shown in Table II, the cells produced 10-20 ng/ml of Factor VIII total antigen as determined by ELISA, and at 12 hrs., 7 ng/ml of Factor VIII was biologically active. However, by 24 hours, the biological activity was lost. The lower level of biologically active Factor VIII at 12 hours and the lack of active Factor VIII at 24 hours can be explained by the fact that the cells were undergoing a cytopathic effect that started at 12

hours and was complete by 24 hours. Therefore, *de novo* synthesis of Factor VIII had probably begun to decrease at 12 hours and the Factor VIII present in the medium was becoming degraded by 24 hours.

D. In Vivo Expression of BDD Factor VIII From Av1ALH81

To determine if human BDD Factor VIII was expressed from Av1ALH81 *in vivo*, and if so, to follow the time course of Factor VIII expression, 15 C57bl/6 female mice were injected with Av1ALH81. The virus was diluted in injection medium (IMEM + 1% FBS) to a total volume of 0.5 ml. Five mice received a dose of 1 x 10^{10} pfu (67 μl of virus; concentration of 1.5 x 10^{11} pfu/ml), five mice received a dose of 4 x 10^{9} pfu (27 μl virus) and five mice received 1 x 10^{9} pfu (7 μl virus). The viral suspension was infused via tail vein over a ten second period using a 0.5 ml syringe and a 27 gauge needle. The control mouse received no injection. One mouse that received 1 x 10^{10} pfu of Av1ALH81 died two days after injection. Blood was taken from each mouse at one week intervals and analyzed for the presence of human Factor VIII antigen by ELISA. The results of the analysis, for the first five weeks post injection, is displayed in Table III below, and graphically in Figure 43.

Table III

In Vivo Expression of Factor VIII

Mouse	Virus Dose	ELISA (Factor VIII ng/ml)				
		Week 1	Week 2	Week 3	Week 4	Week 5
1	$1x10^{10}$	53.3	19.3	0.0	4.3	0.0
2	$1x10^{10}$	45.9	54.6	0.0	34.3	0.0
3	$1x10^{10}$	34.5	35.3	3.0	2.6	0.0
4	$1x10^{10}$	33.1	31.1	10.9	7.7	1.8
Mean	-	41.7	35.1	3.5	12.2	0.5
5	$4x10^{9}$	18.9	7.3	0.0	14.9	1.1
6	$4x10^{9}$	13.0	6.2	5.1	7.9	3.1
7	$4x10^{9}$	9.8	5.2	3.1	12.4	5.1
8	$4x10^{9}$	25.9	13.1	3.4	18.4	12.9
9	$4x10^{9}$	17.1	5.9	0.5	9.7	6.0
Mean	-	16.9	7.5	2.3	12.6	5.6
10	$1x10^{9}$	0.8	0.0	0.0	3.6	0.8
11	$1x10^{9}$	0.4	0.0	0.5	5.1	0.3
12	1.10^{9}	1.1	1.6	2.2	4.2	2.5
13	$1x10^{9}$	1.0	1.7	0.0	1.7	1.3
14	$1x10^{9}$	1.4	0.9	0.0	1.9	0.0
Mean	-	0.9	0.9	0.5	3.3	1.0
Control	0	0.0	0.0	0.0	0.0	0.0

The mice receiving the highest viral dose (1×10^{10} pfu) were producing 42 and 35 ng/ml human BDD Factor VIII one and two weeks post injection, respectively. If these values are corrected for the difference in half-life of human Factor VIII in mice (4-5 hrs., see above) compared to humans (10-12 hrs.), levels in the plasma are adjusted to 126 and 96 ng/ml of Factor VIII at one and two weeks, respectively. Physiological levels of Factor VIII in humans is ~100-200 ng/ml and therapeutic levels are ~10 ng/ml. Therefore, these mice are producing physiological levels of

Factor VIII. In addition, the mice that received the lower dose of 4 x 10^9 pfu of Av1ALH81 are producing Factor VIII protein well over therapeutic levels. The expression of human Factor VIII, in an animal model, has never before been demonstrated.

Three to five weeks post injection, however, the Factor VIII levels had decreased significantly. To determine if this decrease in Factor VIII expression at three weeks was due to an immunological response to the Factor VIII antigen, a second half-life study was performed using full-length human Factor VIII protein. The four mice that had received the highest viral dose (1 x 10^{10} pfu), and four control mice were injected, via tail vein, with 500 ng of full-length human Factor VIII. Blood was drawn at 1, 2, 4, 8, and 12 hours post injection and analyzed for the presence of human Factor VIII antigen by ELISA. Figures 44 and 45 display the results of this analysis. The half-life of full-length Factor VIII is similar in both sets of mice, and can be calculated to be about 3.0 hrs. Two conclusions can be drawn from these data: 1) The half-life of full-length Factor VIII and BDD Factor VIII in mouse plasma are comparable, 3 hrs. and 4-5 hrs., respectively, and 2) the loss in Factor VIII expression in the mice at 3 weeks is not due to rapid clearance of the Factor VIII by specific antibodies in mouse plasma, and, therefore, may be due to the loss of vector from the liver (or loss of the cells containing the vector), or reduced transcription of the Factor VIII cDNA.

The time course of Factor VIII expression in mice was repeated, and the data are shown in Figure 46.

Example 8

Fifteen C57b1/6 female mice were injected via tail vein 4x10^9 pfu (27 μl of virus) of Av1ALH81 in injection medium (IMEM+ 1% FBS). The viral suspension was infused via the tail vein over a ten second period using a 0.5 ml syringe and a 27 gauge needle. A control mouse received no injection. Blood was taken from each

mouse at one week intervals and analyzed for the presence of human Factor VIII antigen by ELISA. The results are shown graphically in Figure 46.

Example 9

Construction of Adenoviral Vectors Including Factor IX Sequences with Genomic Elements

Vectors were prepared in which the Factor IX sequences incorporated genomic elements, i.e., sequences from the human Factor IX gene. These elements included the 5' untranslated region, a centrally truncated first intron, the full 3' untranslated region and naturally-occurring polyadenylation site. The 5' genomic elements were obtained by PCR amplication using genomic Factor IX clones as templates. The three prime untranslated region was obtained from a plasmid provided by Dr. Hsueh (Shanghai, China). An alternative approach, which can be used to readily obtain these elements, is to PCR amplify them from human genomic DNA.

A Factor IX sequence, which includes 9 bp of the Factor IX promoter, the 5' untranslated region, the coding region, and a 162 bp segment of the 3' untranslated region, was excised from pKG5IX-2 (obtained from George Brownlee, University of Oxford, Oxford England) as a Bam HI to HindIII fragment. This fragment is described further in Anson, et al., Nature, Vol. 315, pgs. 683-685 (1985). This insert was inserted into the polylinker of pBluescript II SK+ (Stratagene) to form BLSKH9CI. (Figure 47). The Factor IX sequences were sequenced completely and verified to be correct. Factor IX DNA with genomic elements could also have been obtained according to the procedures disclosed in U.S. Patent No. 4,994,371 and European Patent EP 0 107 278 B1.

A fragment containing the downstream part of the coding sequence, the full 3' untranslated region, the native Factor IX polyadenylation signal, and 331 bp past the polyadenylation site

was excised from pCMVIXa (provided by Jerry Hsueh, Fudan University, Shanghai, China) with PpuMI and BglII. The BglII single strand overhang was blunted. pBLSKH9CI was cut with PpuMI and HindIII, the HindIII site was blunted, and the backbone fragment was joined to the fragment obtained from pCMVIXa as a PpuMI-blunt ligation. The resulting plasmid, pBLSKH9D (Figure 48), contains the 9 bp of promoter, 5' untranslated region, the entire Factor IX coding sequence, the full 3' untranslated region, natural polyadenylation signal, and 331 bp downstream from the polyadenylation signal.

To generate constructs that contain a centrally truncated first intron, a cloning intermediate was prepared. This intermediate removed a Bc1I site downstream from the coding sequence to enable cloning into an upstream Bc1I site. The 3' end of the cDNA in pB1SKH9CI was removed from Ppu MI to HindIII. The single strand overhangs in the plasmid backbone were blunted and ligated to yield pBLH9CINT. (Figure 49)

The 5' end of the cDNA in pBLH9CINT was modified to contain the centrally truncated first intron with a 3 way ligation using 2 PCR generated fragments. These fragments were generated using phage preps as templates (Yoshitake, S, et al., 1985, Biochemistry: 24, 3736-3750). The two PCR generated fragments contained (5' to 3'):

1) SpeI site, SalI site, full 5' untranslated region, first exon of the Factor IX gene, the first 991 bp of the Factor IX first intron, and an AatII site.
2) AatII site, the last 448 bp of the Factor IX first intron, and part of the Factor IX second exon extending past the naturally occurring Bc1I site in the upstream part of this exon.

PCR fragment 1 was digested with SpeI and AatII. PCR fragment 2 was digested with AatII and Bc1I. BLH9CINT was digested with SpeI and Bc1I and the backbone fragment was isolated. The three fragments were joined with a 3 way ligation to yield the plasmid pBLH9EINT. (Figure 50) This plasmid

contains the 5' untranslated region of Factor IX, the first exon, the centrally truncated first intron, and the coding sequence up to the PpuMI site.

To generate pBLH9E (Figure 51), the 3' end of the coding sequence was re-inserted. The 3' end of the Factor IX sequence was excised from pBLSKH9CI and inserted into the pBLH9EINT backbone as an AvaI-AvaI fragment. The resulting plasmid pBLH9E (Figure 51) contained the Factor IX 5' untranslated region, first exon, centrally truncated first intron, remainder of the coding sequence, and 162 bp of 3' untranslated region.

To generate pBLH9F (Figure 52), a fragment containing the 3' end of the coding sequence and the full 3' untranslated region was excised from pBLSKH9D and inserted into the pBLH9EINT backbone as an AvaI-AvaI fragment. Thus pBLH9F has the 5' untranslated region, first exon, truncated first intron, remainder of the coding sequence, full 3' untranslated region, and 300 bp downstream from the polyadenylation site.

The Factor IX sequences were then excised from pBLSKH9D, pBLH9E, and pBLH9F and inserted into the pAvS6 backbone as SpeI-ClaI fragments. The resulting plasmids were termed pAV1H9D (Figure 53), pAV1H9E, and pAV1H9F, respectively. However, when pAv1H9E and pAv1H9F were sequenced, errors were found in the 5' untranslated region of the Factor IX gene. These errors were repaired. The sequence errors were traced back to pBLH9EINT. Miniprep one this plasmid had been used to generate the subsequent plasmids. pBLH9EINT miniprep six was found to have the correct sequence. The SpeI to AatII fragment in pBLH9EINT miniprep six was used to replace the corresponding fragment in pAv1H9E and pAv1H9F to yield pAv1H9ER (Figure 54) and pAv1H9FR (Figure 55), respectively. These plasmids contain the adenovirus type 5 ITR, RSV promoter, tripartite leader, Factor IX sequence, SV40 polyadenylation site (which is superfluous in pAV1H9D and pAV1H9FR), and adenovirus homologous recombination region.

pAV1H9D, pAV1H9ER, and pAV1H9FR were then used to generate adenoviral vectors by procedures hereinabove described. Briefly,

linearized plasmids were co-transfected with the large ClaI-cut fragment of Ad dl327 into 293 cells. Plaques were selected, expanded, and screened. The three vector isolates chosen were termed Ad1H9D, Ad1H9ER, Ad1H9FR. These were grown into large scale preps and plaque titered on 293 cells.

Example 10

2×10^8 pfu of Av1H9B, Ad1H9D, Ad1H9ER, or Ad1H9FR were injected via tail vein into C57B1/6 mice. Two mice received each virus. One week later, plasma was sampled by ELISA and an immunochromogenic bioassay described above for human Factor IX antigen and biological activity, respectively. The ELISA results, shown in Figure 56, demonstrate that the inclusion of genomic elements dramatically increased Factor IX expression. The Ad1H9FR vector effected the most expression which was more than 200 fold greater than the level obtained with Av1H9B. The immunochromogenic assay results for eight experimental mice and one negative control mouse (which was injected with the beta-galactosidase vector, Av1LacZ4) are shown in Table IV below.

Table IV

Vector	Mouse Number	Plasma Factor IX (ng/ml)	
		ELISA	Immunochromogenic
Av1H9B	1	<8	<8
Av1H9B	2	<8	<8
Ad1H9D	3	74	65
Ad1H9D	4	48	49
Ad1H9ER	5	116	102
Ad1H9ER	6	101	80
Ad1H0FR	7	1,339	1,051
Ad1H9FR	8	1,467	1,391
Av1LacZ4	9	<8	<8

These results demonstrate that the human Factor IX expressed from Ad1H9D, Ad1H9ER, and Ad1H9FR was functional. Livers were collected one week after vector injection and DNA and RNA were prepared. Southern analysis demonstrated an average of 1-2 vector copies per liver cell for all four vectors. (Data not shown.) Northern analysis revealed RNA species of the correct size for each vector with band intensities that parallelled the Factor IX plasma levels. (Data not shown.)

Example 11

1×10^9 pfu of Av1H9B, Ad1H9D, or Ad1H9ER were injected via tail vein into C57B1/6 mice. 5×10^7 pfu of Ad1H9ER and Ad1H9FR were also administered to C57B1/6 mice via tail vein injection. The cohort size for each regimen was 5 mice. At the indicated time points plasma was obtained and assayed for human Factor IX by ELISA. The results, shown in Figures 57 and 58, again demonstrate that the inclusion of genomic elements in the Factor IX sequences dramatically increased Factor IX expression. Factor IX levels that approach normal were obtained with the low dose of 1×10^9 pfu of Ad1H9D and Ad1H9ER. Injection of a very low dose

of 5 x 10^7 pfu of Ad1H9FR resulted in the expression of potentially therapeutic levels of human Factor IX in the mice. In each case, high-level expression from Ad1H9D, Ad1H9ER, and Ad1H9FR persisted for the 10-12 week duration of the experiment. This exceeded the 7 week duration of expression which had previously been achieved with higher doses of Av1H9B.

Example 12

Expression from Av1H9B, Ad1H9D, and Ad1H9ER was tested in tissue culture. HepG2 and HeLa cells were transduced at an moi of 2 (2 pfu per cell). 48 hours later the medium was collected and assayed by ELISA for human Factor IX. The data, shown in Figure 59, demonstrate that the incremental improvements seen in HepG2 cells with Ad1H9D and Ad1H9ER correspond to those seen *in vivo* in mice that received 2 x 10^8 pfu of virus. In HeLa cells, the inclusion of the 3' untranslated region (Ad1H9D) had no effect, whereas inclusion of the intron (Ad1H9ER) improved expression dramatically.

All patents, publications, and database entries referenced in this specification are indicative of the level of skill of persons in the art to which the invention pertains. The disclosures of all such patents, publications (including published patent applications), and database entries are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication, and database entry was specifically and individually indicated to be incorporated by reference.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

WHAT IS CLAIMED IS:

1. An adenoviral vector including at least one DNA sequence encoding a clotting factor.

2. The vector of Claim 1 wherein said DNA sequence encodes Factor VIII or a fragment, derivative, or analogue thereof having Factor VIII clotting activity.

3. The vector of Claim 1 wherein said DNA sequence encodes Factor IX or a fragment, derivative, or analogue thereof having Factor IX clotting activity.

4. A plasmid vector for generating an adenoviral vector, said plasmid vector including at least one DNA sequence encoding a clotting factor.

5. A method of treating hemophilia in a host, comprising:

administering to said host the adenoviral vector of Claim 1, said vector being administered in an amount effective to treat hemophilia in said host.

6. The vector of Claim 2 wherein said vector further includes a tissue-specific promoter.

7. The vector of Claim 6 wherein said tissue-specific promoter is the mouse albumin promoter.

8. The vector of Claim 3 wherein said vector further includes a promoter which is not a tissue-specific promoter.

9. The vector of Claim 8 wherein said promoter which is not a tissue-specific promoter is a Rous Sarcoma Virus promoter.

10. The vector of Claim 3 wherein said vector further includes at least one genomic element.

11. The vector of Claim 10 wherein said genomic element is the full 3' untranslated region of the Factor IX DNA sequence.

12. The vector of Claim 11 wherein said vector further includes the full 5' untranslated region of the Factor IX DNA sequence.

13. The vector of Claim 10 wherein said vector includes the full 3' untranslated region of the Factor IX DNA sequence, the full 5' untranslated region of the Factor IX DNA sequence, and a centrally truncated first intron of the Factor IX gene.

14. The vector of Claim 10 wherein said vector further includes the full seventh intron of the Factor IX gene.

15. The vector of Claim 2 wherein said vector further includes at least one genomic element.

16. The vector of Claim 15 wherein said vector includes the ApoA1 promoter.

17. The vector of Claim 16 wherein said vector further includes the first intron of the apolipoprotein A-1 gene.

18. The vector of Claim 16 wherein said vector further includes the first exon of the apolipoprotein A-1 gene.

19. The vector of Claim 16 wherein said vector further includes the first intron of the apolipoprotein A-1 gene and the first exon of the apolipoprotein A-1 gene.

20. An adenoviral vector including at least one DNA sequence encoding a heterologous protein, and at least one genomic element affecting expression of said at least one DNA sequence encoding a heterologous protein.

FIG. 1

CONSTRUCTION STRATEGY FOR THE pG1 VECTOR BACKBONE

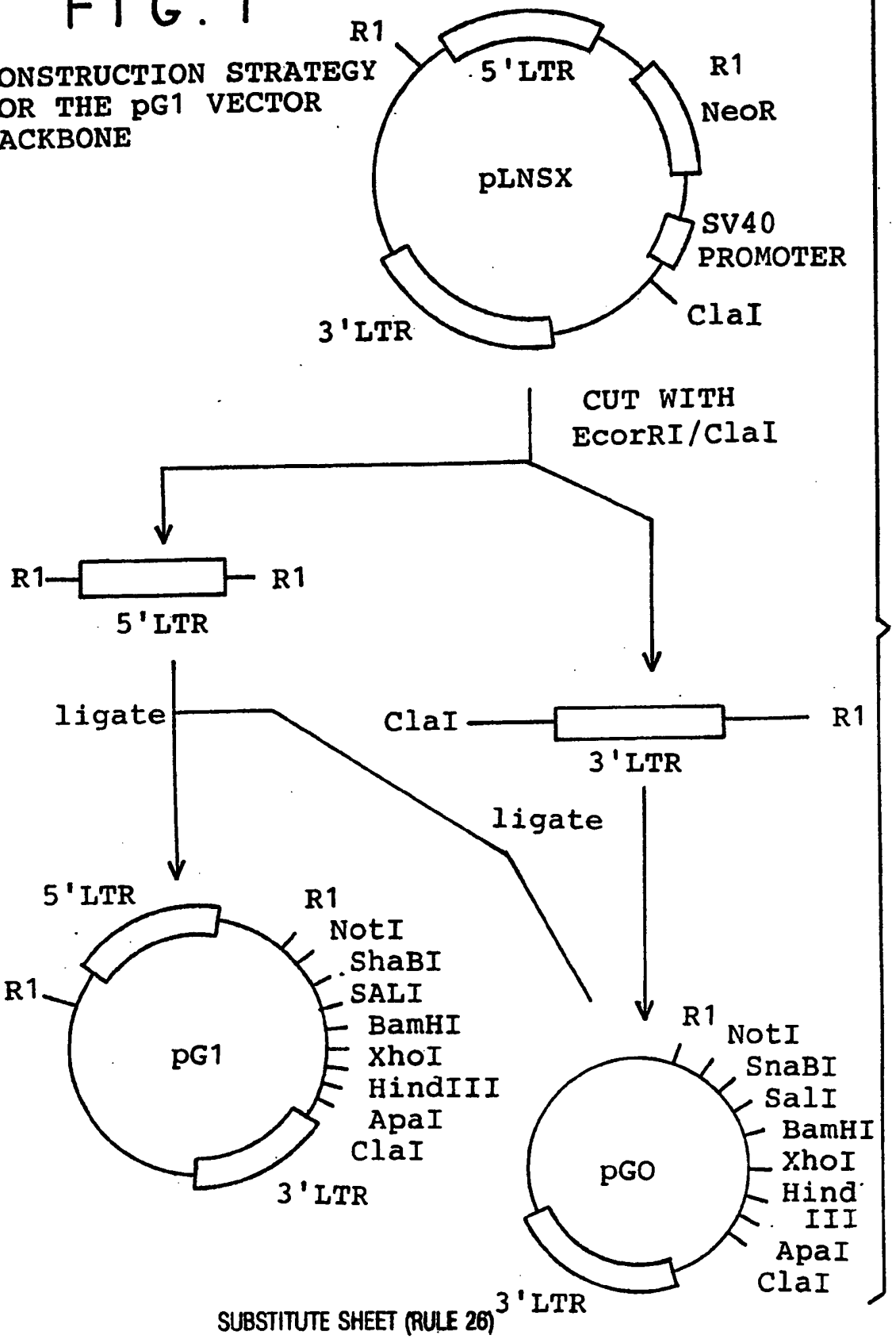

FIG. 2

SEQUENCE OF THE MULTIPLE CLONING SITE IN THE pGI PLASMID

1/2 EcoRI	NotI	SnaBI	SalI	BamHI	XhoI	HindIII	ApaI
AATTC	GCGGCCGC	TACGTA	GTCGAC	GGATCC	CTCGAG	AAGCTT	GGGCCC
G	CGCCGGCG	ATGCAT	CAGCTG	CCTAGG	GAGCTC	TTCGAA	CCCGGG

1/2ClaI
AT
TAGC

FIG. IIA

1	AGGTTATGCA	GCGCGTGAAC	ATGATCATGG	CAGAATCACC	AGGCCTCATC	ACCATCTGCC
61	TTTTAGGATA	TCTACTCAGT	GCTGAATGTA	CAGTTTTTCT	TGATCATGAA	AACGCCAACA
121	AAATTCTGAA	TCGGCCAAAG	AGGTATAATT	CAGGTAAATT	GGAAGAGTTT	GTTCAAGGGA
181	ACCTTGAGAG	AGAATGTATG	GAAGAAAAGT	GTAGTTTTGA	AGAAGCACGA	GAAGTTTTTG
241	AAAACACTGA	AAGAACAACT	GAATTTTGGA	AGCAGTATGT	TGATGGAGAT	CAGTGTGAGT
301	CCAATCCATG	TTTAAATGGC	GGCAGTTGCA	AGGATGACAT	TAATTCCTAT	GAATGTTGGT
361	GTCCCTTTGG	ATTTGAAGGA	AAGAACTGTG	AATTAGATGT	AACATGTAAC	ATTAAGAATG
421	GCAGATGCGA	GCAGTTTTGT	AAAAATAGTG	CTGATAACAA	GGTGGTTTGC	TCCTGTACTG
481	AGGGATATCG	ACTTGCAGAA	AACCAGAAGT	CCTGTGAACC	AGCAGTGCCA	TTTCCATGTG
541	GAAGAGTTTC	TGTTTCACAA	ACTTCTAAGC	TCACCCGTGC	TGAGACTGTT	TTTCCTGATG
601	TGGACTATGT	AAATTCTACT	GAAGCTGAAA	CCATTTTGGA	TAACATCACT	CAAAGCACCC

MATCH WITH FIG. IIB

MATCH WITH FIG. IIA

FIG. IIB

```
661   AATCATTTAA TGACTTCACT CGGGTTGTTG GTGGAGAAGA TGCCAAACCA GGTCAATTCC
721   CTTGGCAGGT TGTTTTGAAT GGTAAAGTTG ATGCATTCTG TGGAGGCTCT ATCGTTAATG
781   AAAAATGGAT TGTAACTGCT GCCCACTGTG TTGAAACTGG TGTTAAAATT ACAGTTGTCG
841   CAGGTGAACA TAATATTGAG GAGACAGAAC ATACAGAGCA AAAGCGAAAT GTGATTCGAA
901   TTATTCCTCA CCACAACTAC AATGCAGCTA TTAATAAGTA CAACCATGAC ATTGCCCTTC
961   TGGAACTGGA CGAACCCTTA GTGCTAAACA GCTACGTTAC ACCTATTTGC ATTGCTGACA
1021  AGGAATACAC GAACATCTTC CTCAAATTTG GATCTGGCTA TGTAAGTGGC TGGGGAAGAG
1081  TCTTCCACAA AGGGAGATCA GCTTTAGTTC TTCAGTACCT TAGAGTTCCA CTTGTTGACC
1141  GAGCCACATG TCTTCGATCT ACAAAGTTCA CCATCTATAA CAACATGTTC TGTGCTGGCT
1201  TCCATGAAGG AGGTAGAGAT TCATGTCAAG GAGATAGTGG GGGACCCCAT GTTACTGAAG
1261  TGGAAGGGAC CAGTTTCTTA ACTGGAATTA TTAGCTGGGG TGAAGAGTGT GCAATGAAAG
1321  GCAAATATGG AATATATACC AAGGTATCCC GGTATGTCAA CTGGATTAAG GAAAAAACAA
1381  AGCTCACTTA ATGAAAGATG GATTTCCAAG GTTAATTCAT TGGAATTGAA AATTAACAGG
1441  GCCTCTCACT AACTAATCAC TTTCCCATCT TTTGTTAGAT TTGAATATAT ACATTCTATG
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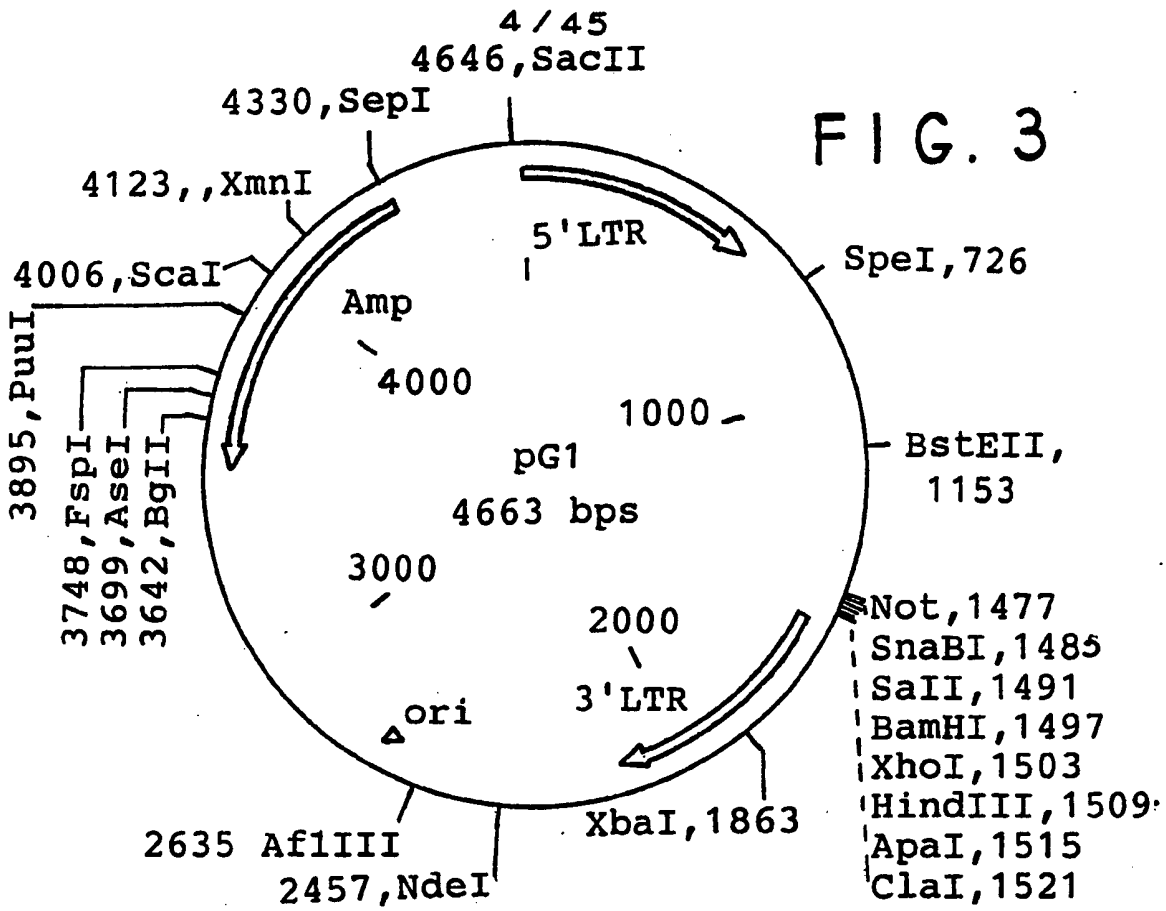
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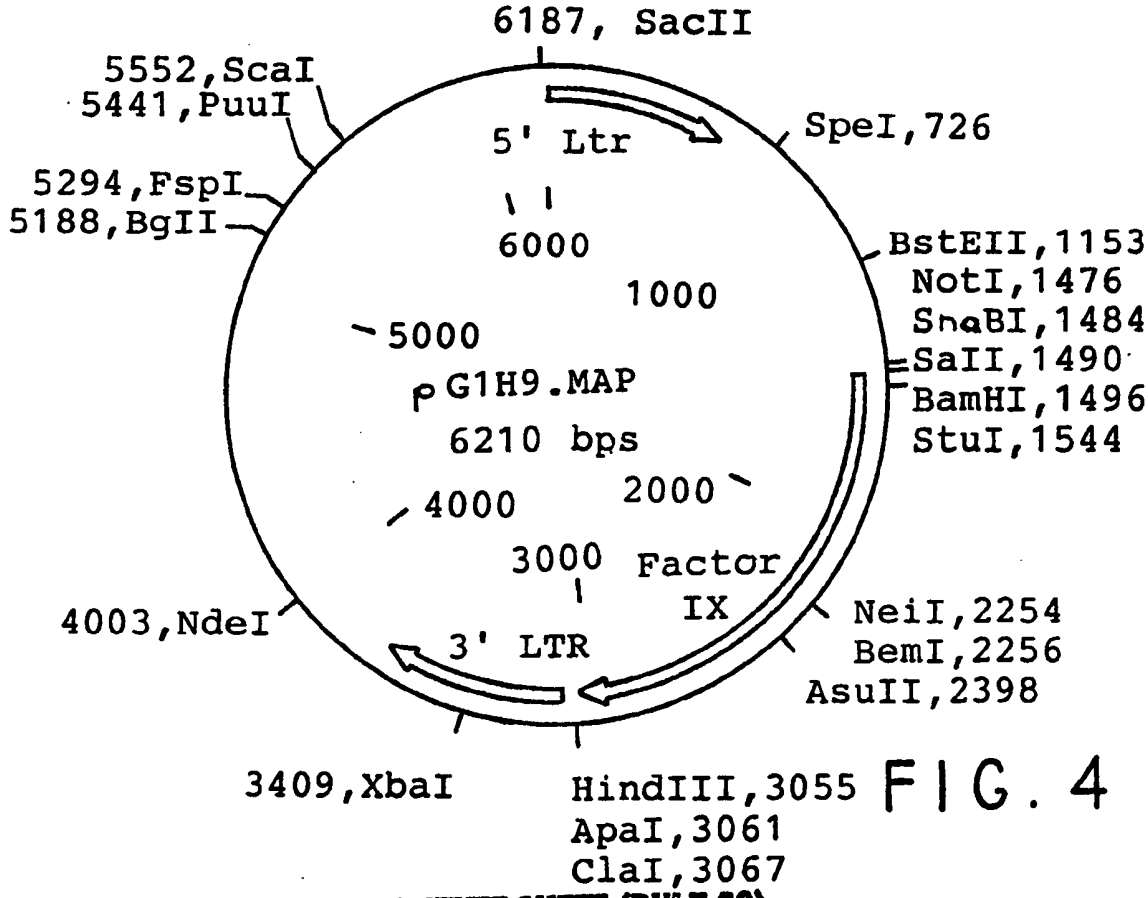
FIG. 3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IX
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SalI,1490
BamHI,1496
StuI,1544
NeiI,2254
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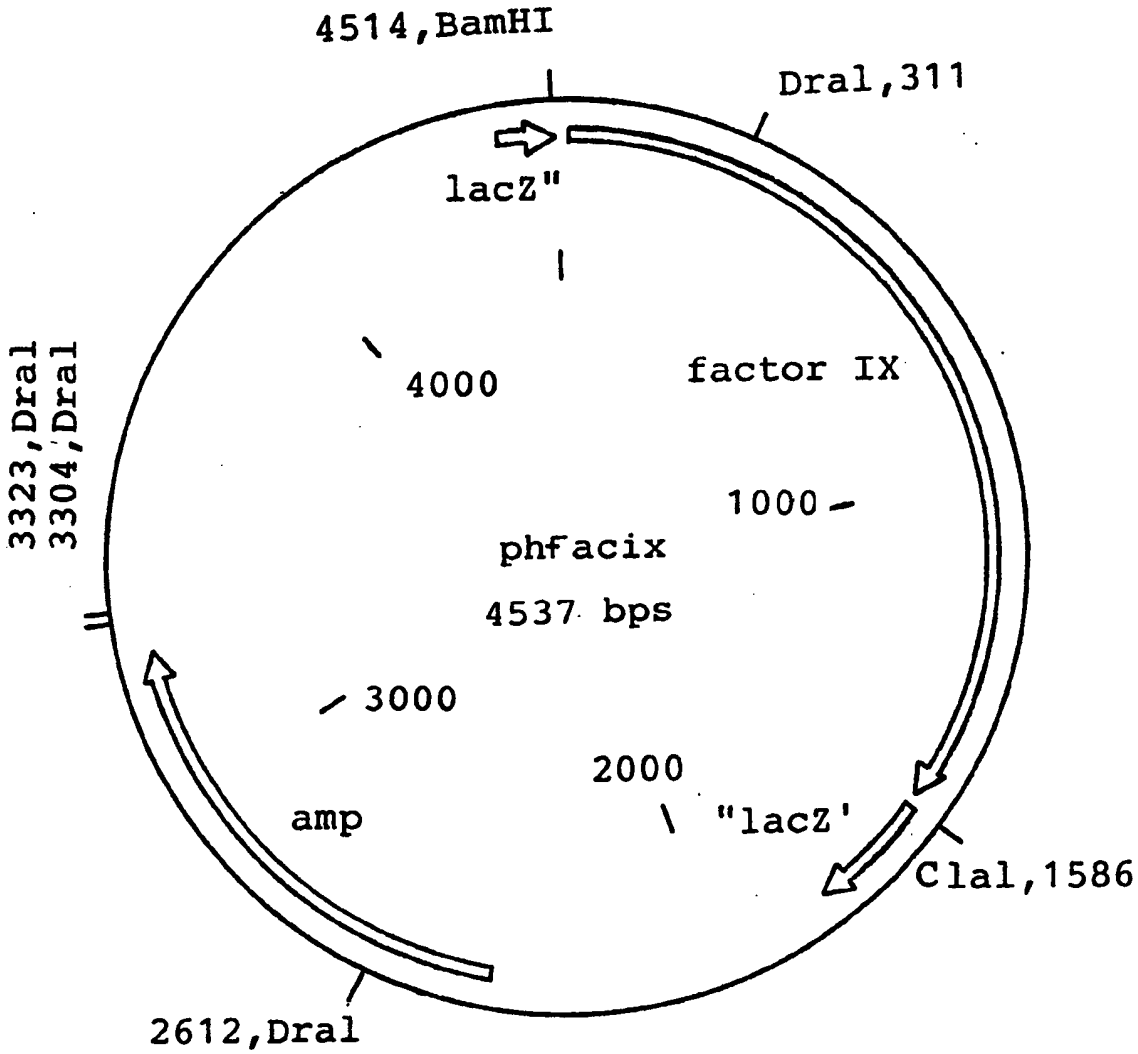
FIG. 4

FIG. 5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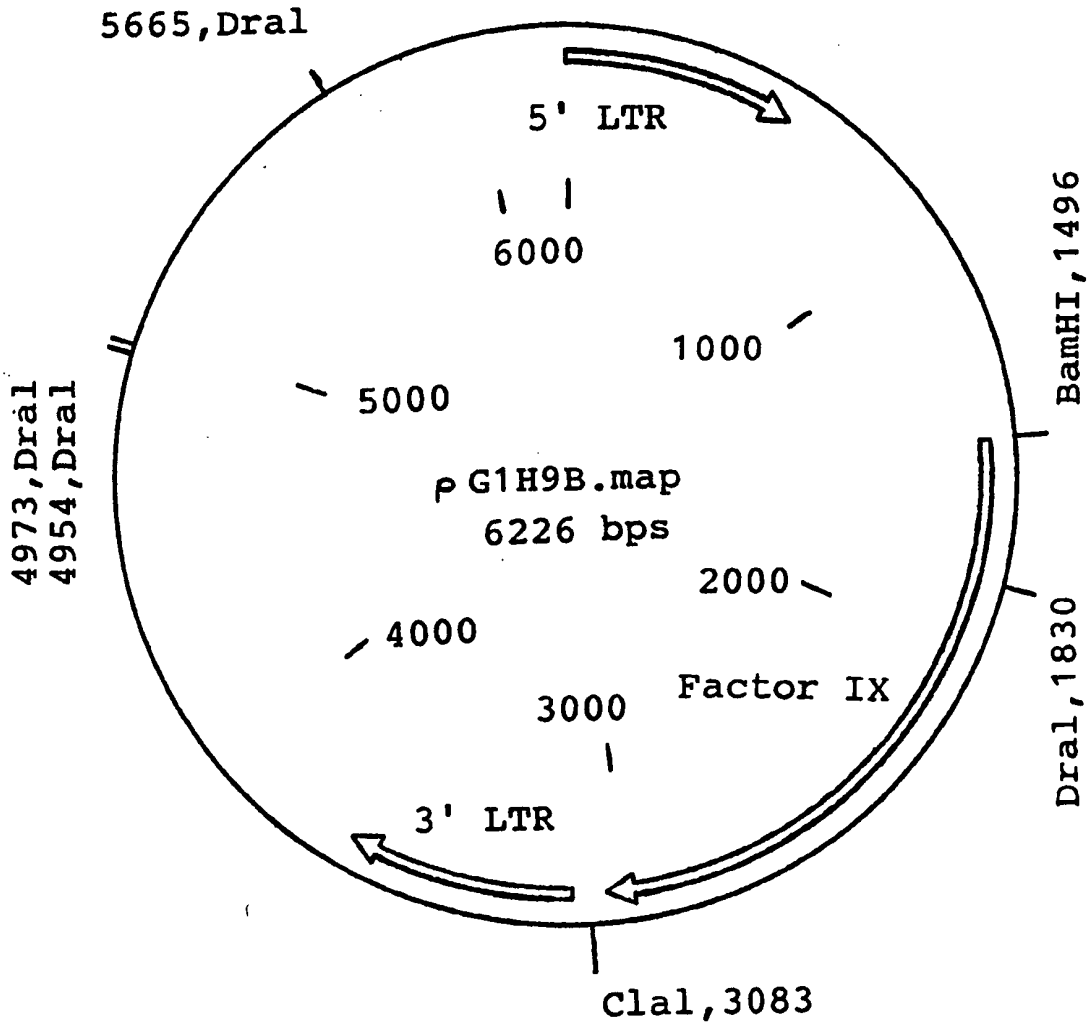
3323,DraI
3304,DraI

FIG. 6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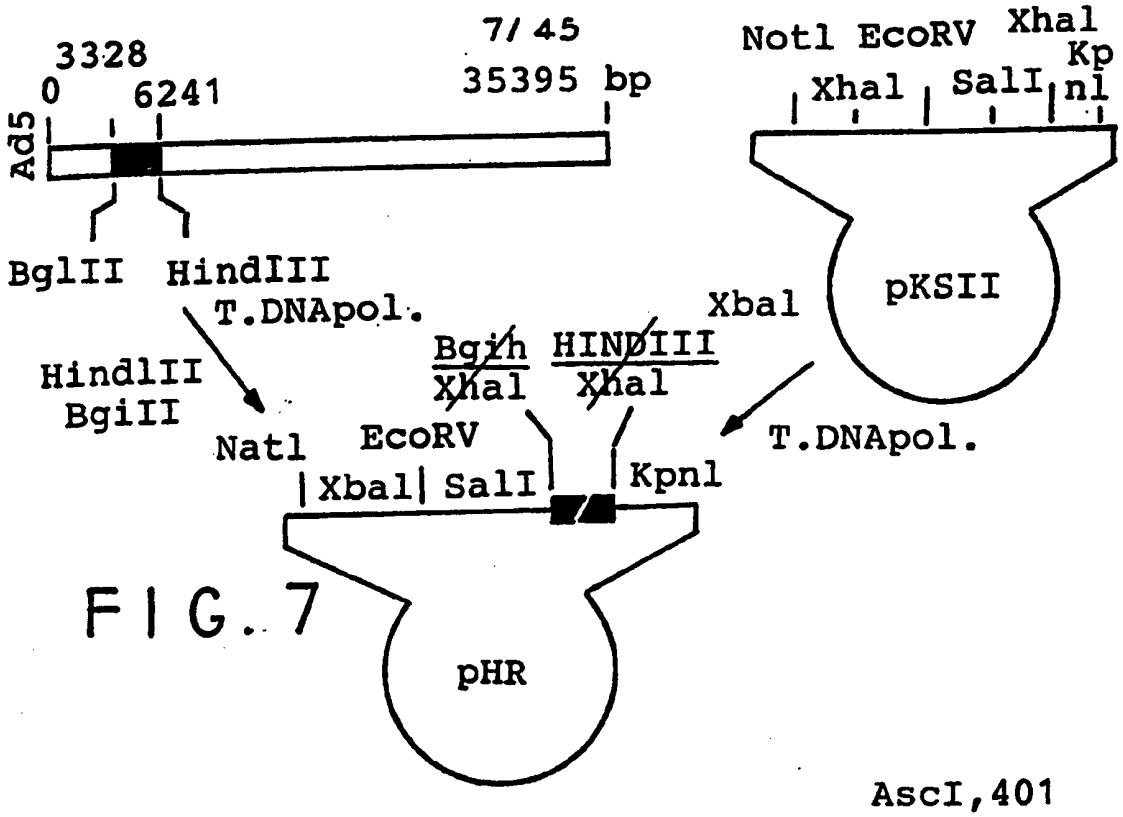
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BglII
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SalI
BglII
XhaI
HINDIII
XhaI
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pHR
NotI EcoRV XhaI
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SalI
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T.DNApol.

FIG. 7

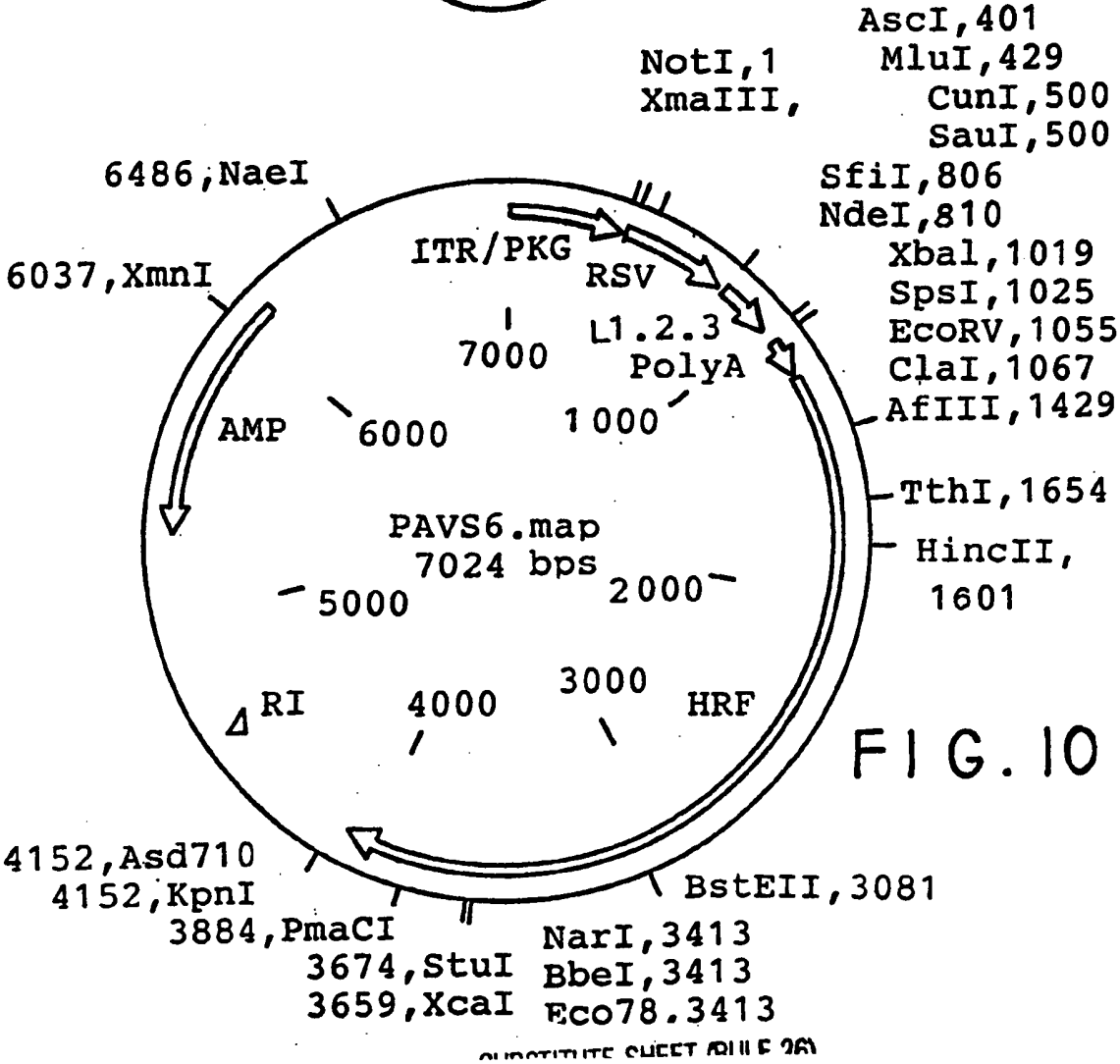
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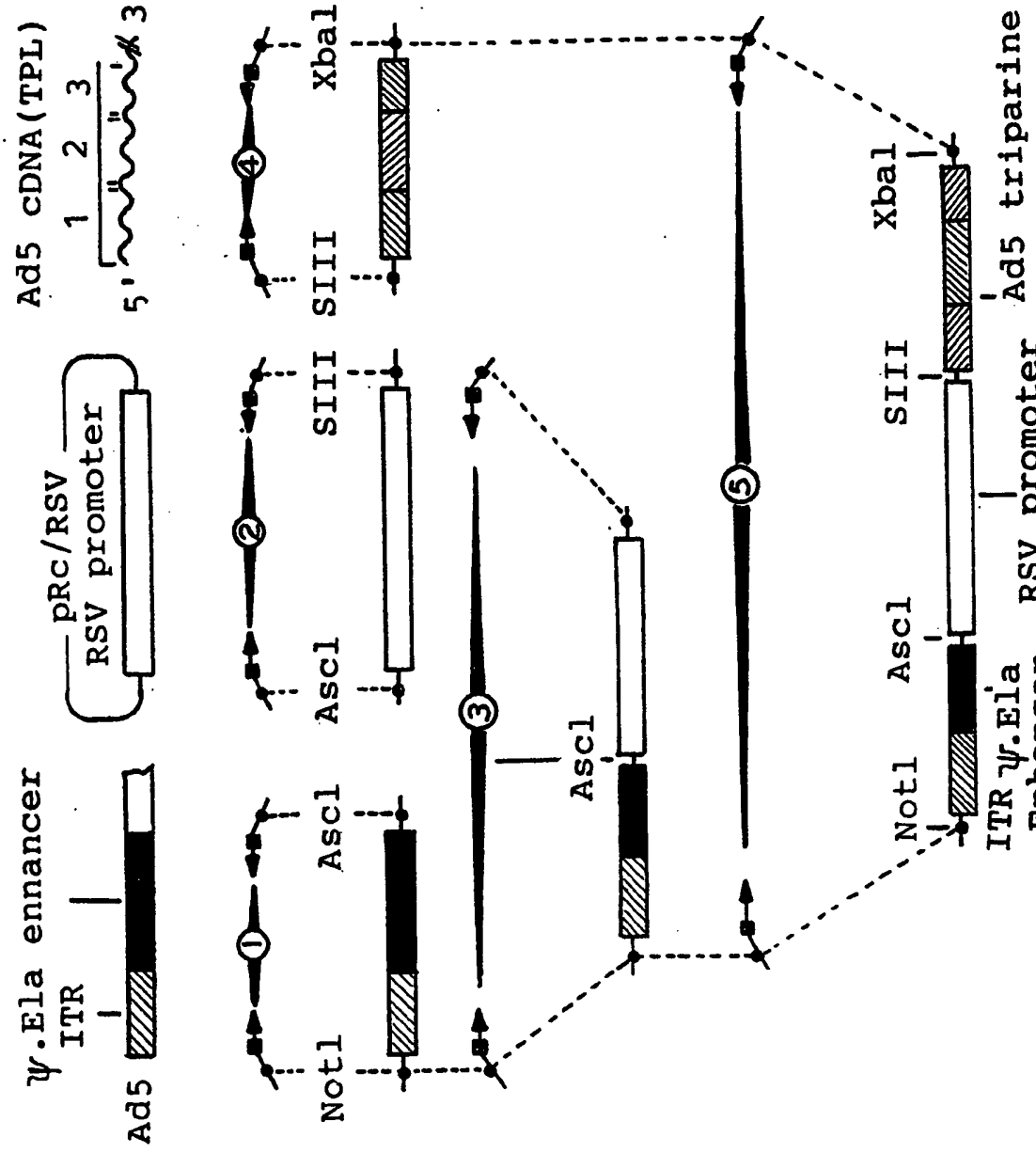
FIG. 10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5'
3'
pRc/RSV
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ψ.Ela ennancer
ITR
Ad5
NotI
AscI
AscI
SIII
XbaI
AscI
AscI
SIII
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SIII
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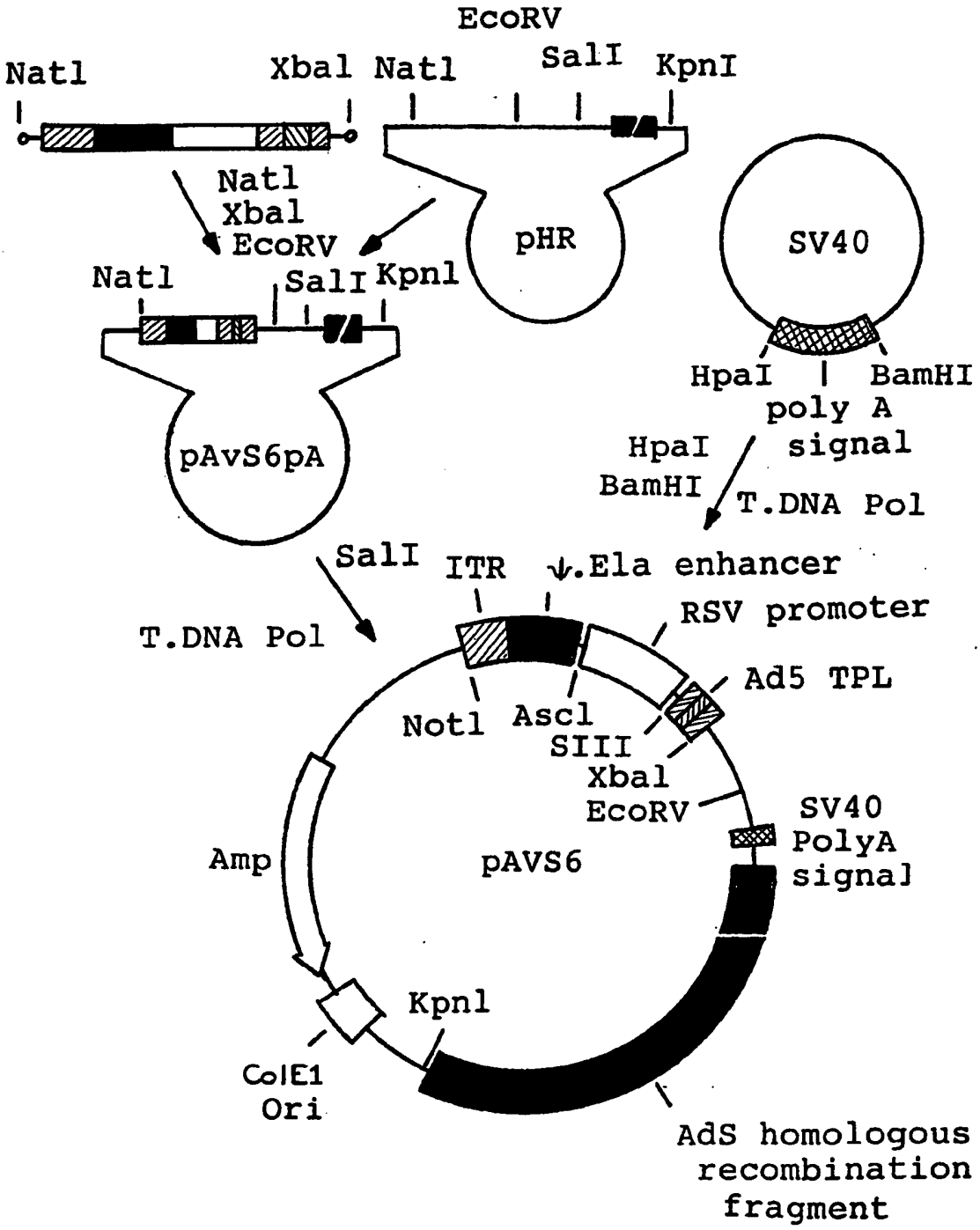
RSV promoter
Ad5 triparine leader

FIG. 8

FIG. 9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poly A
signal
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HpaI
BamHI
T.DNA Pol
SalI
ITR
Ela enhancer
T.DNA Pol
RSV promoter
Ad5 TPL
NotI
AscI
SIII
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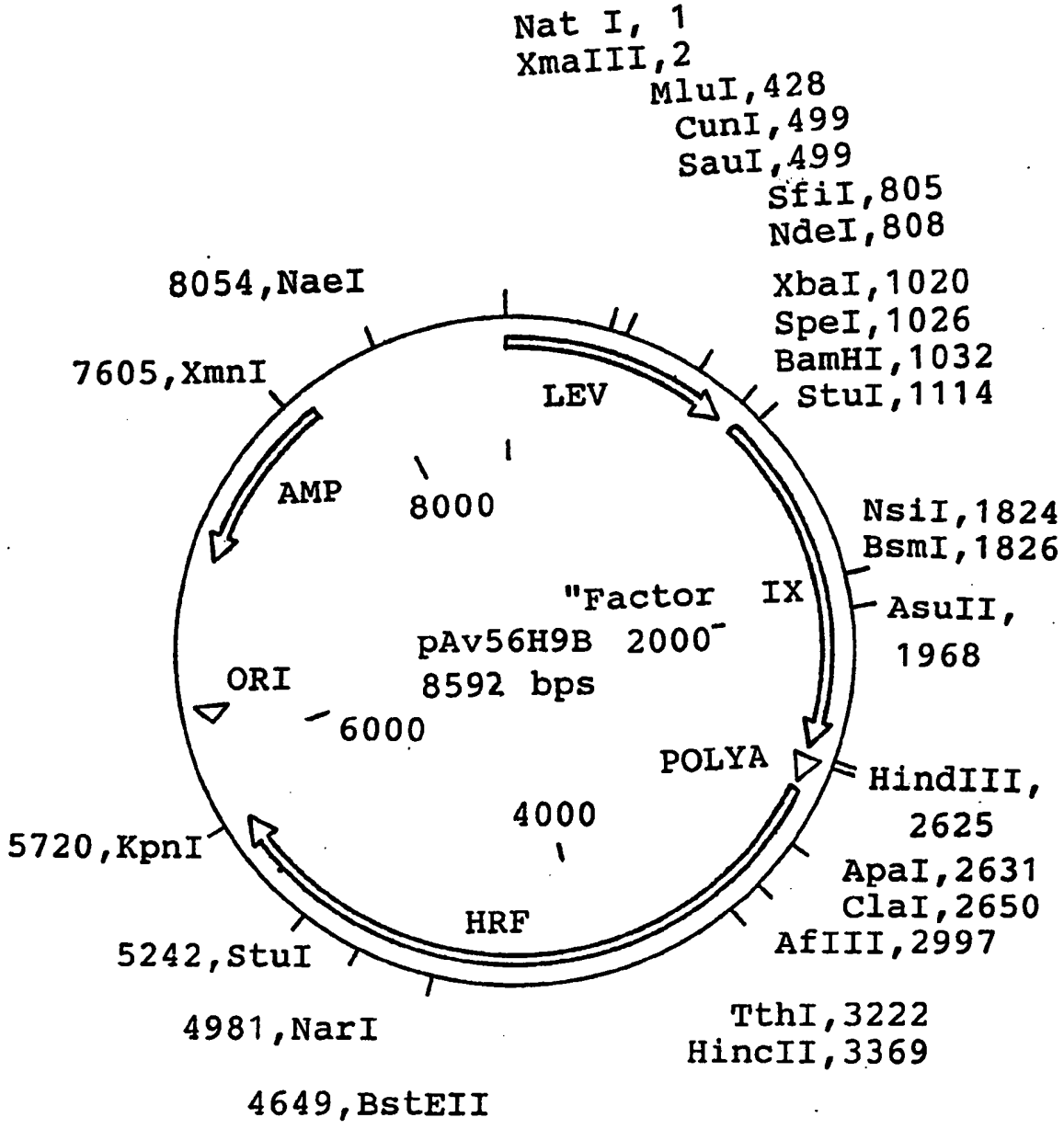
Ori
AdS homologous
recombination
fragment

FIG. 12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ClaI,2650
AfIII,2997
TthI,3222
HincII,3369
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4981,NarI
5242,StuI
5720,KpnI
7605,XmnI
8054,NaeI
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ORI
POLYA
HRF
"Factor IX
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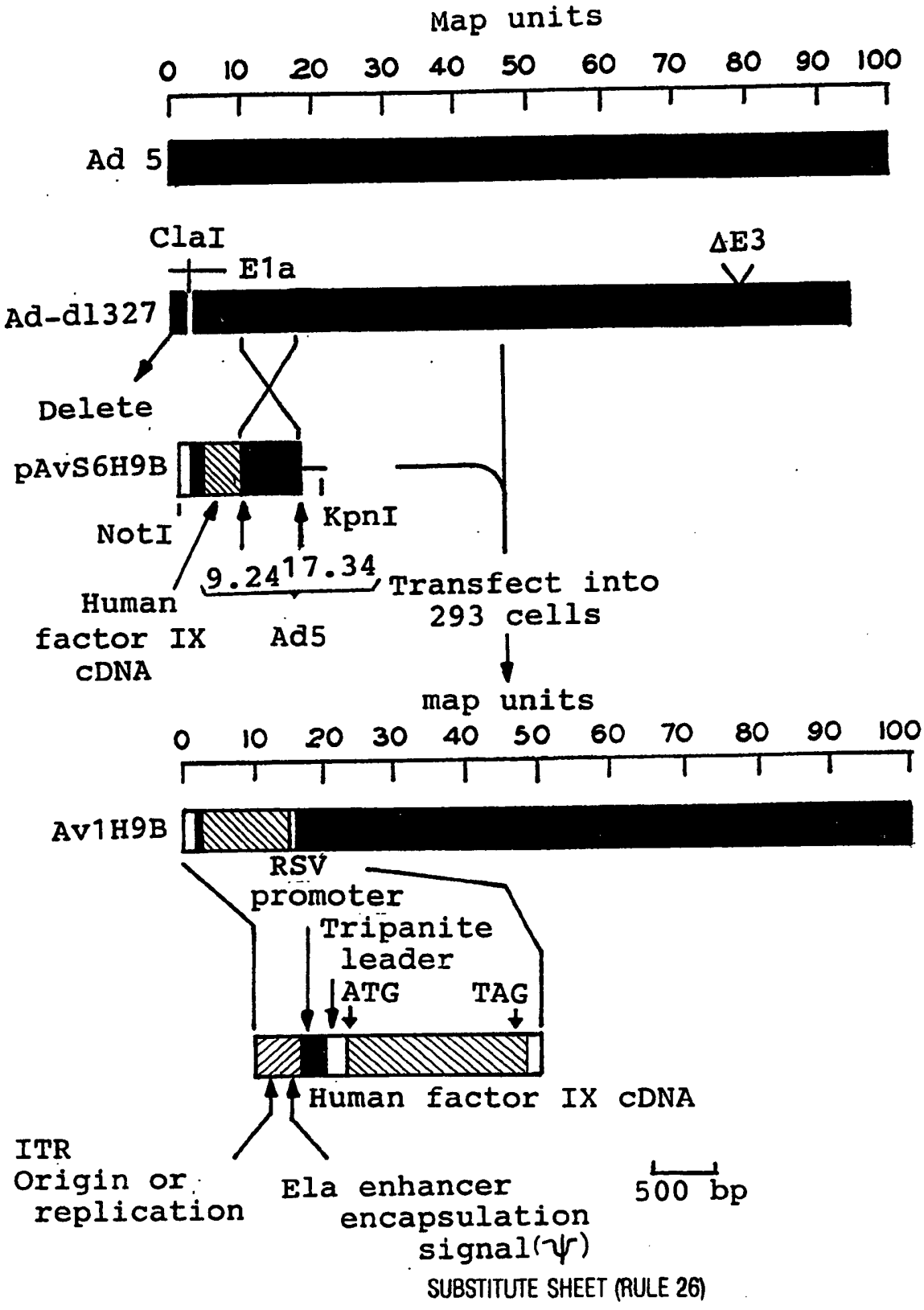
6000
4000

FIG. 13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Ad-dl327
Delete
pAvS6H9B
NotI
KpnI
9.24
17.34
Human factor IX cDNA
Ad5
Transfect into 293 cells
map units
0 10 20 30 40 50 60 70 80 90 100
Av1H9B
RSV promoter
Tripanite leader
ATG
TAG
Human factor IX cDNA
ITR Origin or replication
Ela enhancer encapsulation signal(ψ)
500 bp

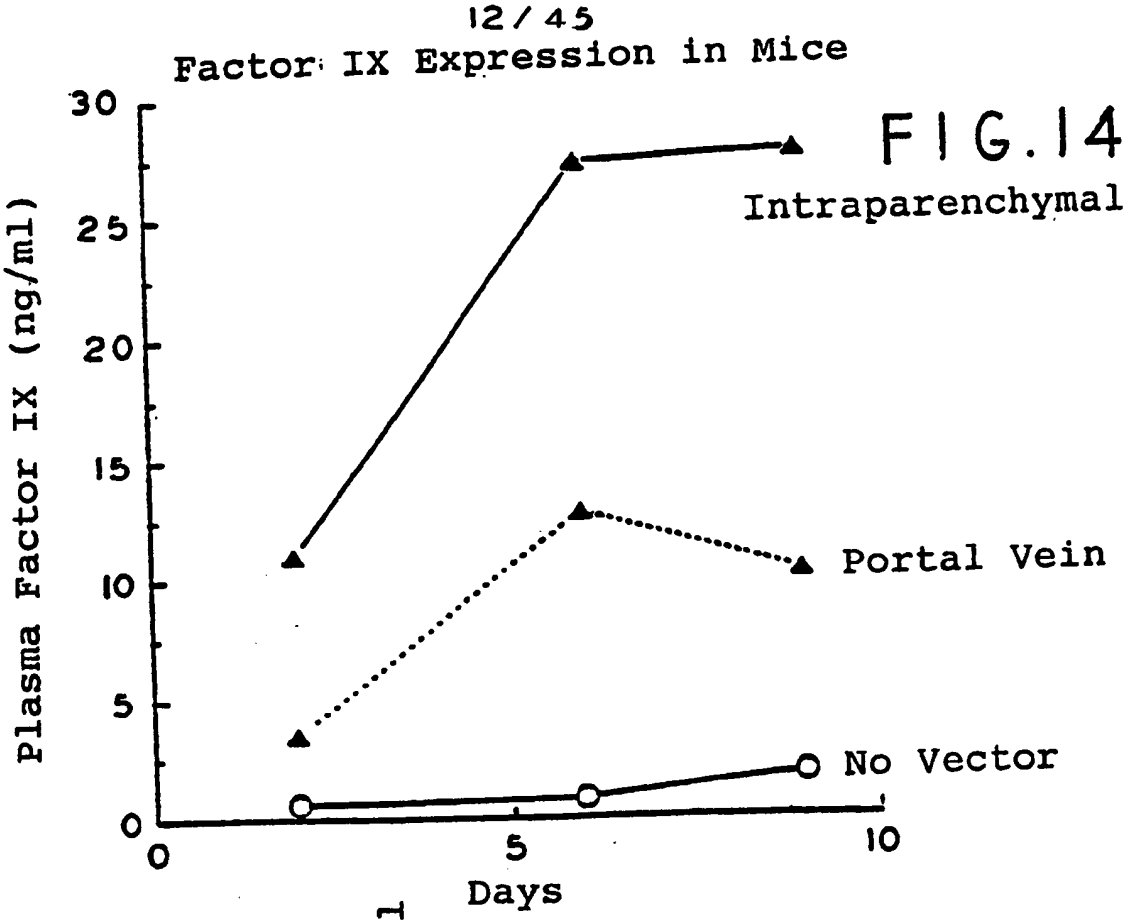

Factor IX Expression in Mice
Plasma Factor IX (ng/ml)
30
25
20
15
10
5
0
0
5
10
Days
Intraparenchymal
Portal Vein
No Vector

FIG. 14

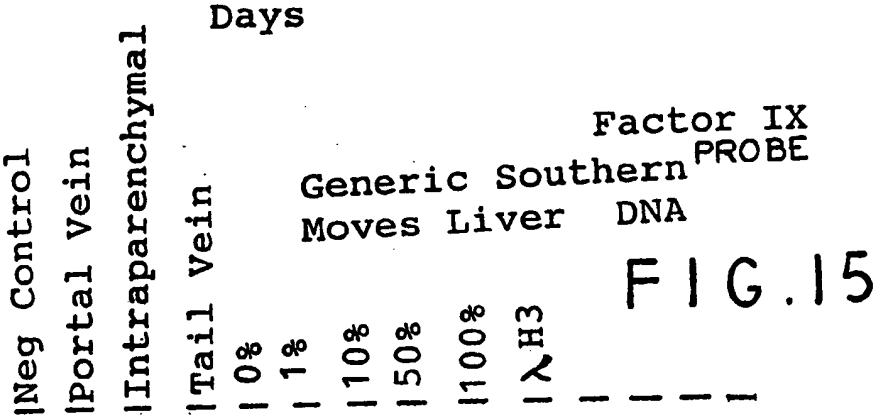

Factor IX PROBE
Generic Southern
Moves Liver DNA
Neg Control
Portal Vein
Intraparenchymal
Tail Vein
0%
1%
10%
50%
100%
λH3
23.1
9.4
6.6
4.4
2.7
2.0

FIG. 15

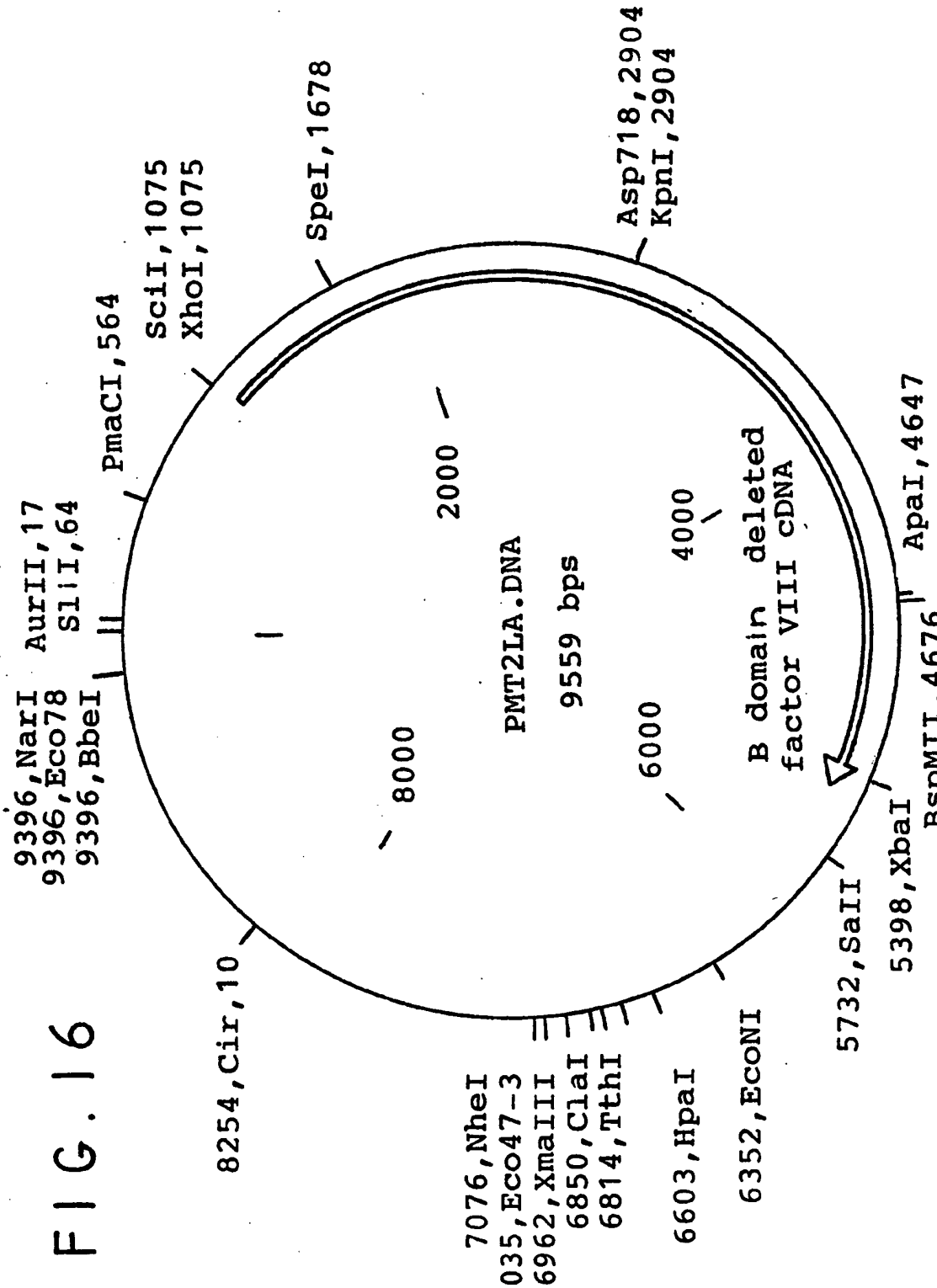

PMT2LA.DNA
9559 bps
1
2000
4000
6000
8000
B domain deleted
factor VIII cDNA
Asp718,2904
KpnI,2904
SpeI,1678
SclI,1075
XhoI,1075
PmaCI,564
AurII,17
S1II,64
9396,NarI
9396,Eco78
9396,BbeI
8254,Cir,10
7076,NheI
7035,Eco47-3
6962,XmaIII
6850,ClaI
6814,TthI
6603,HpaI
6352,EcoNI
5732,SaII
5398,XbaI
BspMII,4676
ApaI,4647

FIG. 16

FIG. 17A

```
   1  ATGCAAATAG AGCTCTCCAC CTGCTTCTTT CTGTGCCTTT TGCGATTCTG CTTTAGTGCC
  61  ACCAGAAGAT ACTACCTGGG TGCAGTGGAA CTGTCATGGG ACTATATGCA AAGTGATCTC
 121  GGTGAGCTGC CTGTGGACGC AAGATTTCCT CCTAGAGTGC CAAAATCTTT TCCATTGAAC
 181  ACCTCAGTCG TGTACAAAAA GACTCTGTTT GTAGAATTCA CGGTTCACCT TTTCAACATC
 241  GCTAAGCCAA GGCCACCCTG GATGGGTCTG CTAGGTCCTA CCATCCAGGC TGAGGTTTAT
 301  GATACAGTGG TCATTACACT TAAGAACATG GCTTCCCATC CTGTCAGTCT TCATGCTGTC
 361  GGTGTATCCT ACTGGAAAGC TTCTGAGGGA GCTGAATATG ATGATCAGAC CAGTCAAAGG
 421  GAGAAAGAAG ATGATAAAGT CTTCCCTGGT GGAAGCCATA CATATGTCTG GCAGGTCCTG
 481  AAAGAGAATG GTCCAATGGC CTCTGACCCA CTGTGCCTTA CCTACTCATA TCTTTCTCAT
 541  GTGGACCTGG TAAAAGACTT GAATTCAGGC CTCATTGGAG CCCTACTACT ATGTAGAGAA
 601  GGGAGTCTGG CCAAGGAAAA GACACAGACC TTGCACAAAT TTATACTACT TTTTGCTGTA
 661  TTTGATGAAG GGAAAAGTTG GCACTCAGAA ACAAAGAACT CCTTGATGCA GGATAGGGAT
 721  GCTGCATCTG CTCGGGCCTG GCCTAAAATG CACACAGTCA ATGGTTATGT AAACAGGTCT
 781  CTGCCAGGTC TGATTGGATG CCACAGGAAA TCAGTCTATT GGCATGTGAT TGGAATGGGC
 841  ACCACTCCTG AAGTGCACTC AATATTCCTC GAAGGTCACA CATTTCTTGT GAGGAACCAT
 901  CGCCAGGCGT CCTTGGAAAT CTCGCCAATA ACTTTCCTTA CTGCTCAAAC ACTCTTGATG
 961  GACCTTGGAC AGTTTCTACT GTTTTGTCAT ATCTCTTCCC ACCAACATGA TGGCATGGAA
1021  GCTTATGTCA AAGTAGACAG CTGTCCAGAG GAACCCCAAC TACGAATGAA AAATAATGAA
1081  GAAGCGGAAG ACTATGATGA TGATCTTACT GATCTCGAAA TGGATGTGGT CAGGTTTGAT
1141  GATGACAACT CTCCTTCCTT TATCCAAATT CGCTCAGTTG CCAAGAAGCA TCCTAAAACT
1201  TGGTGACATT ACATTGCTGC TGAAGAGGAG GACTGGGACT ATGCTCCCTT AGTCCTCGCC
1261  CCCGATGACA GAAGTTATAA AAGTCAATAT TTGAACAATG GCCCTCAGCG GATGGGTAGG
1321  AAGTACAAAA AAGTCCGATT TATGGCATAC ACAGATGAAA CCTTTAAGAC TCGTGAAGCT
1381  ATTCAGCATG AATCAGGAAT CTTGGGACCT TTACTTTATG GGGAAGTTGG AGACACACTG
1441  TTGATTATAT TTAAGAATCA AGCAAGCAGA CCATATAACA TCTACCCTCA CGGAATCACT
1501  GATGTCCGTC CTTTGTATTC AAGGAGATTA CCAAAAGGTG TAAAACATTT GAAGGATTTT
```

MATCH WITH FIG. 17B

MATCH WITH FIG. 17A

FIG. 17B

1561	CCAATTCTGC	CAGGAGAAAT	ATTCAAATAT	AAATGGACAG	TGACTGTAGA	AGATGGGCCA
1621	ACTAAATCAG	ATCCTCGGTG	CCTGACCCCC	TATTACTCTA	GTTTCGTTAA	TATGGAGAGA
1681	GATCTAGCTT	CAGGACTCAT	TGGCCCTCTC	CTCATCTGCT	ACAAAGAATT	TGTAGATCAA
1741	AGAGGAAACC	AGATAATGTC	AGACAAGAGG	AATGTCATCC	TGTTTTCTGT	ATTTGATGAG
1801	AACCGAAGCT	GGTACCTCAC	AGAGAATATA	CAACGCTTTC	TCCCCAATCC	AGCTGGAGTG
1861	CAGCTTGAGG	ATCCAGAGTT	CCAAGCCTCC	AACATCATGC	ACAGCATCAA	TGGCTATGTT
1921	TTTGATAGTT	TGCAGTTGTC	AGTTTGTTTC	CATCACCTGG	CATACTGGTA	CATTCTAAGC
1981	ATTGGAGCAC	AGACTGACTT	CCTTTCTGTC	TTCTTCTCTG	GATATACCTT	CAAACACAAA
2041	ATGGTCTATG	AAGACACACT	CACCCTATTC	CCATTCTCAG	GAGAAACTGT	CTTCATGTCG
2101	ATGGAAAACC	CAGGTCTATG	GATTCTGGGG	TGCCACAACT	CAGACTTTCG	GAACAGAGGC
2161	ATGACCGCCT	TACTGAAGGT	TTCTAGTTGT	GACAAGAACA	CTGGTGATTA	TTACGAGGAC
2221	AGTTATGAAG	ATATTTCAGC	ATACTTGCTG	AGTAAAAACA	ATGCCATTGA	ACCAAGAAGC
2281	TTCTCCCAGA	ATTCAAGAGA	CCCTAGCACT	AGGCAAAAGC	AATTTAATGC	CACCCCACCA
2341	GTCTTGAAAC	GCCATCAACG	GGAAATAACT	CGTACTACTC	TTCAGTCAGA	TCAAGAGGAA
2401	ATTGACTATG	ATGATACCAT	ATCAGTTGAA	ATGAAGAAGG	AAGATTTTGA	CATTTATGAT
2461	GAGGATGAAA	ATCAGAGCCC	CCGCAGCTTT	CAAAAGAAAA	CACGACACTA	TTTTATTGCT
2521	GCAGTGGAGA	GGCTCTGGGA	TTATGGGATG	ACTAGCTCCC	CACATGTTGT	AAGAAAGAGG
2581	GCTCAGAGTG	GCAGTGTCCC	TCAGTTCAAG	AAAGTTGTTT	TCCAGGAATT	TACTGATGGC
2641	TCCTTTACTC	AGCCCTTATA	CCGTGGAGAA	CTAAATGAAC	ATTTGGGACT	CCTGGGGCCA
2701	TATATAAGAG	CAGAAGTTGA	AGATAATATC	ATGGTAACTT	TCAGAAATCA	GGCTCTTCGT
2761	CCCTATTCCT	TCTATTCTAG	CCTTATTTCT	TATGAGGAAG	ATCAGAGGCA	AGGAGCAGAA
2821	CCTAGAAAAA	ACTTTGTCAA	GCCTAATGAA	ACCAAAACTT	ACTTTTGGAA	AGTGCAACAT
2881	CATATGGCAC	CCACTAAAGA	TGAGTTTGAC	TGCAAAGCCT	GGGCTTATTT	CTCTGATGTT
2941	GACCTGGAAA	AAGATGTGCA	CTCAGGCCTG	ATTGGACCCC	TTCTGGTCTG	CCACACTAAC
3001	ACACTGAACC	CTGCTCATGG	GAGACAAGTG	ACAGTACAGG	AATTTGCTCT	GTTTTTCACC
3061	ATCTTTGATG	AGACCAAAAG	CTGGTACTTC	ACTGAAAATA	TGGAAAGAAA	CTGCAGGGCT

MATCH WITH FIG. 17C

MATCH WITH FIG. 17B

FIG. 17C

3121	CCCTGCAATA	TCCAGATGGA	AGATCCCACT	TTTAAAGAGA	ATTATCGCTT	CCATGCAATC
3181	AATGGCTACA	TAATGGATAC	ACTACCTGGC	TTAGTAATGG	CTCAGGATCA	AAGGATTCGA
3241	TGGTATCTGC	CTAGCATGGG	GAGCAATGAA	AACATCCATT	CTATTCATTT	CAGTGGACAT
3301	GTGTTCACTG	TACGAAAAAA	AGAGGAGTAT	AAAATGGCAC	TGTACAATCT	CTATCCAGGT
3361	GTTTTTGAGA	CAGTGGAAAT	GTTACCATCC	AAAGCTGGAA	TTTGGCGGGT	GGAATGCCTT
3421	ATTGGCGAGC	ATCTACATGC	TGGGATGAGC	ACACTTTTTC	TGGTGTACAG	CAATAAGTGT
3481	CAGACTCCCC	TGGGAATGGC	TTCTGGACAC	ATTAGAGATT	TTCAGATTAC	AGCTTCAGGA
3541	CAATATGGAC	AGTGGGCCCC	AAAGCTGGCC	AGACTTCATT	ATTCCGGATC	AATCAATGCC
3601	TGGAGCACCA	AGGAGCCCTT	TTCTTGGATC	AAGGTGGATC	TGTTGGCACC	AATGATTATT
3661	CACGGCATCA	AGACCCAGGG	TGCCCGTCAG	AAGTTCTCCA	GCCTCTACAT	CTCTCAGTTT
3721	ATCATCATGT	ATAGTCTTGA	TGGGAAGAAG	TGGCAGACTT	ATCGAGGAAA	TTCCACTGGA
3781	ACCTTAATGG	TCTTCTTTGG	CAATGTGGAT	TCATCTGGGA	TAAAACACAA	TATTTTTAAC
3841	CCTCCAATTA	TTGCTCGATA	CATCCGTTTG	CACCCAACTC	ATTATAGCAT	TCGCAGCACT
3901	CTTCGCATGG	AGTTGATGGG	CTGTGATTTA	AATAGTTGCA	GCATGCCATT	GGGAATGGAG
3961	AGTAAAGCAA	TATCAGATGC	ACAGATTACT	GCTTCATCCT	ACTTTACCAA	TATGTTTGCC
4021	ACCTGGTCTC	CTTCAAAAGC	TCGACTTCAC	CTCCAAGGGA	GGAGTAATGC	CTGGAGACCT
4081	CAGGTGAATA	ATCCAAAAGA	GTGGCTGCAA	GTGGACTTCC	AGAAGACAAT	GAAAGTCACA
4141	GGAGTAACTA	CTCAGGGAGT	AAAATCTCTG	CTTACCAGCA	TGTATGTGAA	GGAGTTCCTC
4201	ATCTCCAGCA	GTCAAGATGG	CCATCAGTGG	ACTTCTTTTT	TTCAGAATGG	CAAAGTAAAG
4261	GTTTTTCAGG	GAAATCAAGA	CTCCTTCACA	CCTGTGGTGA	ACTCTCTAGA	CCCACCGTTA
4321	CTGACTCGCT	ACCTTCGAAT	TCACCCCCAG	AGTTGGTGGC	ACCAGATTGC	CCTGAGGATG
4381	GAGGTTCTGG	GCTGCGAGGC	ACAGGACCTC	TACTGAGGGT	GGCCACTGCA	GCACCTGCCA
4441	CTGCCGTCAC	CTCTCCCTCC	TCAGCTCCAG	GGCAGTGTCC	CTCCCTGGCT	TGCCTTCTAC
4501	CTTTGTGCTA	AATCCTAGCA	GACACTGCCT	TGAAGCCTCC	TGAATTAACT	ATCATCAGTC
4561	CTGCATTTCT	TTGGTGGGGG	GCCAGGAGGG	TGCATCCAAT	TTAACTTAAC	TCTTACCTAT
4621	TTTCTGCAG					

SUBSTITUTE SHEET (RULE 26)

FIG. 18

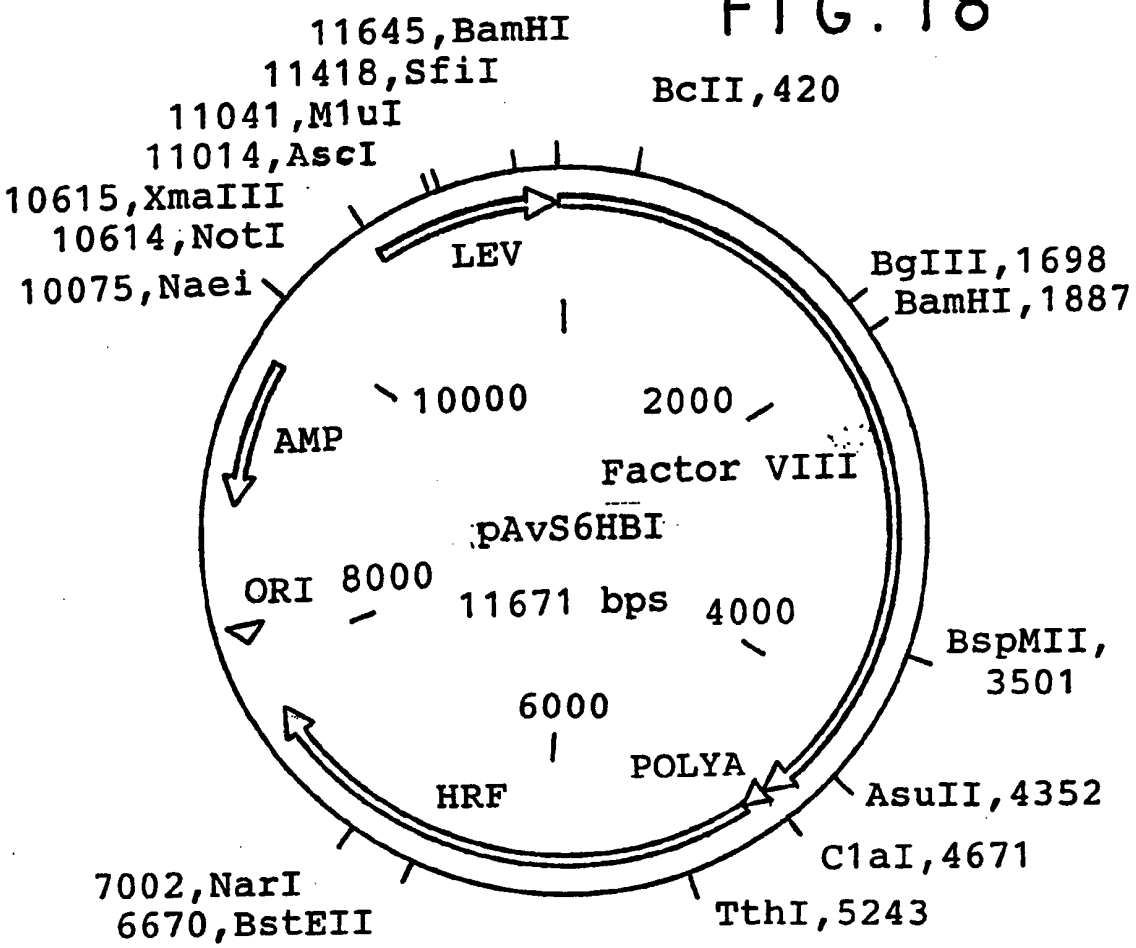

11645,BamHI
11418,SfiI
11041,M1uI
11014,AscI
10615,XmaIII
10614,NotI
10075,NaeI
BcII,420
BgIII,1698
BamHI,1887
LEV
10000
2000
AMP
Factor VIII
pAvS6HBI
ORI
8000
11671 bps
4000
BspMII,
3501
6000
POLYA
HRF
AsuII,4352
C1aI,4671
7002,NarI
6670,BstEII
TthI,5243

FIG. 23

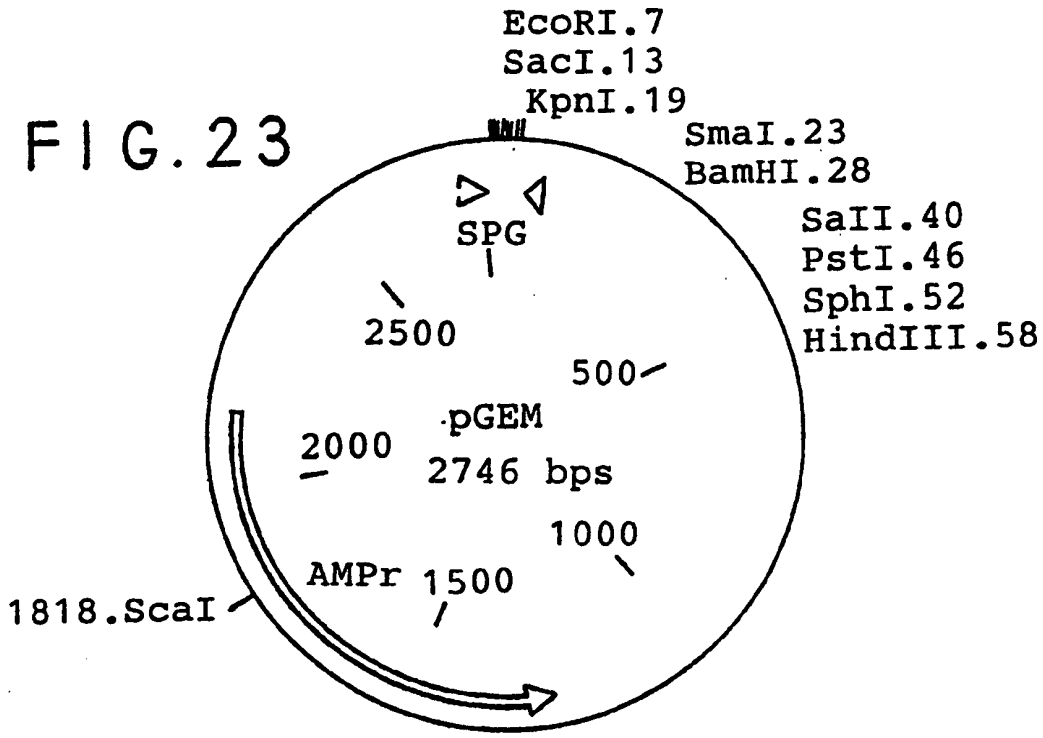

EcoRI.7
SacI.13
KpnI.19
SmaI.23
BamHI.28
SaII.40
PstI.46
SphI.52
HindIII.58
SPG
2500
500
pGEM
2000
2746 bps
1000
AMPr
1500
1818.ScaI

FIG. 19

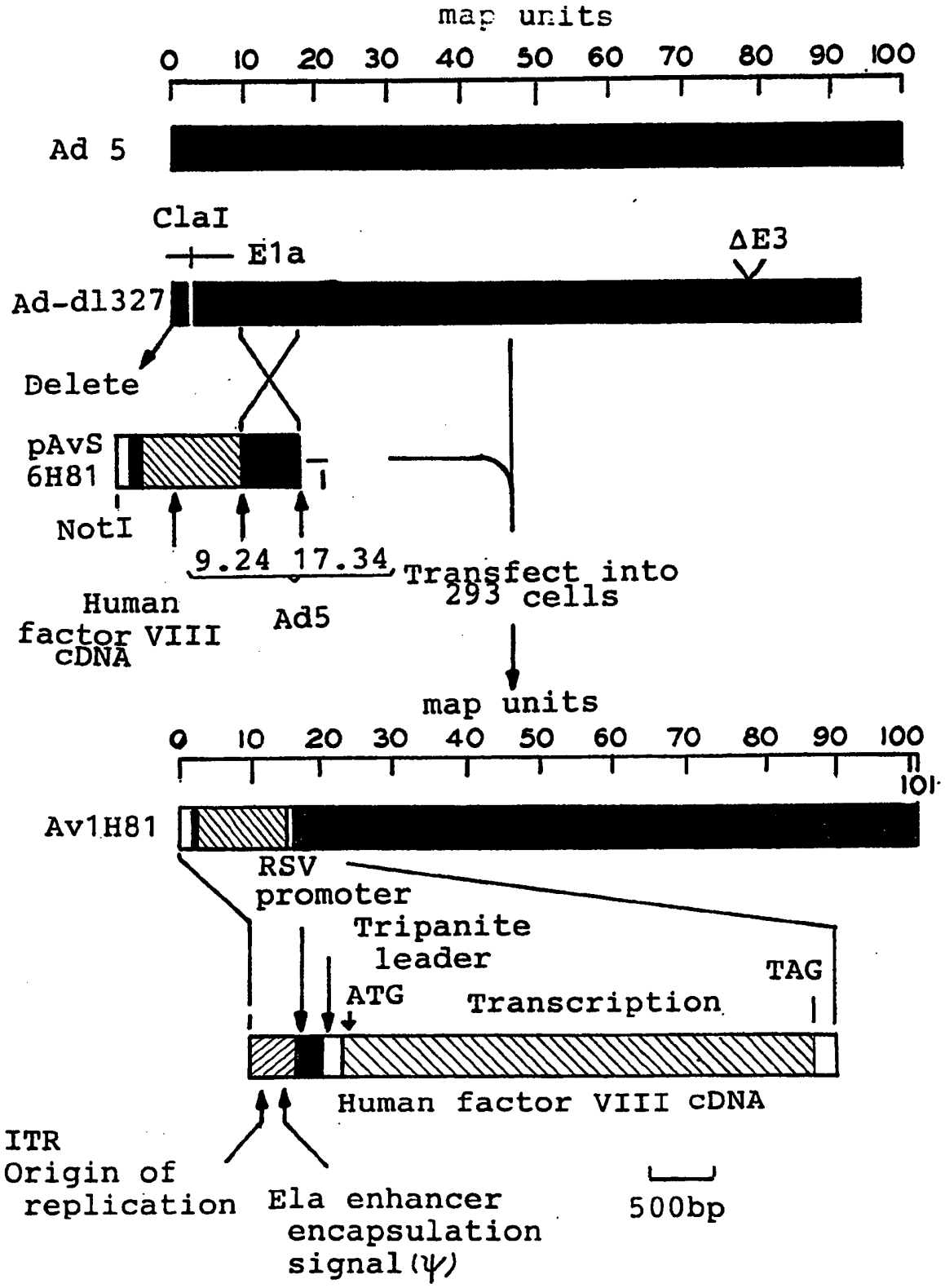

map units
0 10 20 30 40 50 60 70 80 90 100
Ad 5
ClaI
E1a
ΔE3
Ad-dl327
Delete
pAvS
6H81
NotI
9.24 17.34
Transfect into
293 cells
Human
factor VIII
cDNA
Ad5
map units
0 10 20 30 40 50 60 70 80 90 100
101
Av1H81
RSV
promoter
Tripanite
leader
ATG
Transcription
TAG
Human factor VIII cDNA
ITR
Origin of
replication
Ela enhancer
encapsulation
signal (ψ)
500bp

FIG. 20

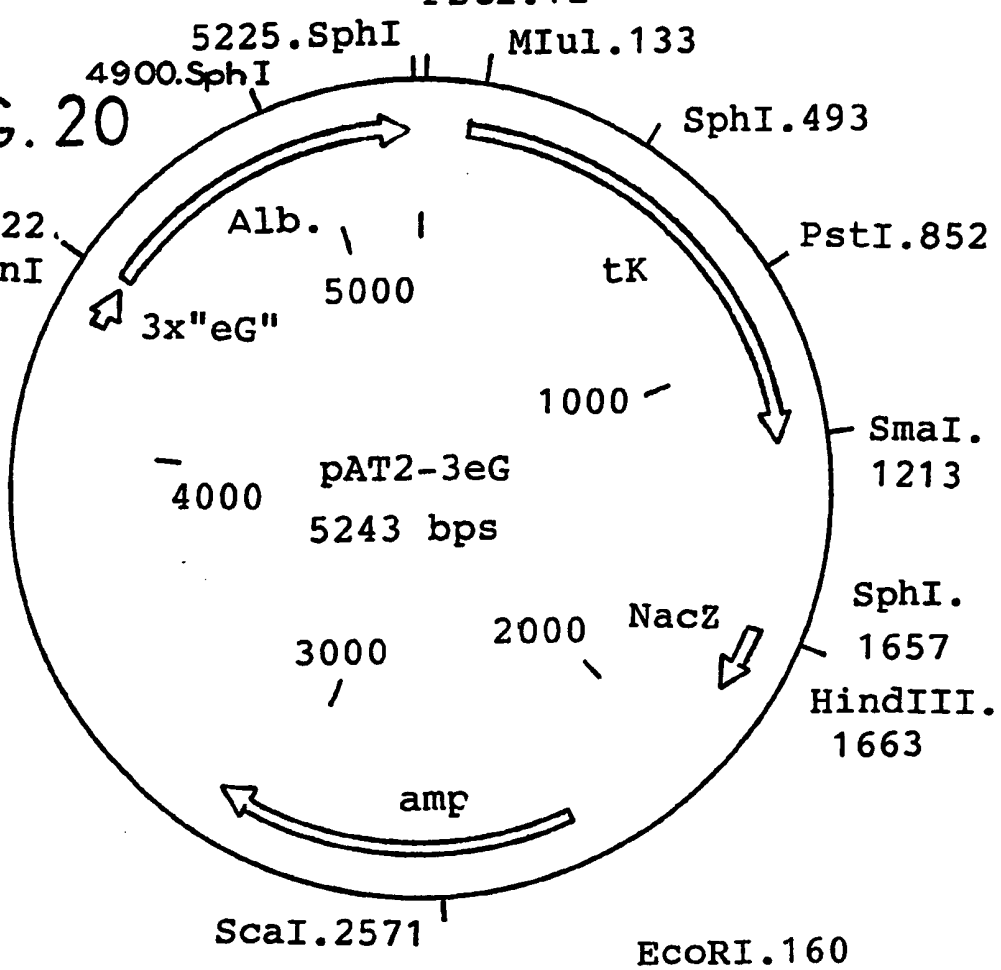

PstI.12
5225.SphI
MIul.133
4900.SphI
SphI.493
4422.
.KpnI
Alb.
5000
tK
PstI.852
3x"eG"
1000
SmaI.
1213
4000
pAT2-3eG
5243 bps
SphI.
1657
2000
NacZ
3000
HindIII.
1663
amp
ScaI.2571

FIG. 31

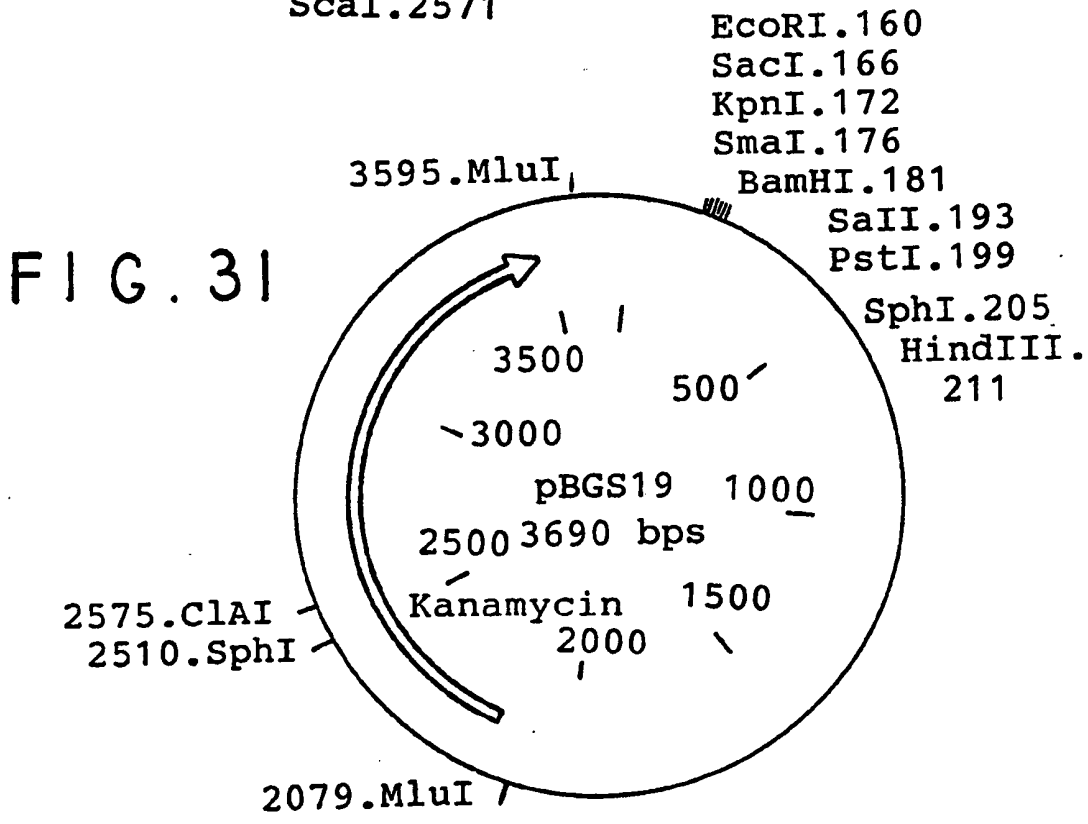

EcoRI.160
SacI.166
KpnI.172
SmaI.176
3595.MluI
BamHI.181
SaII.193
PstI.199
SphI.205
HindIII.
211
3500
500
3000
pBGS19
1000
2500
3690 bps
Kanamycin
1500
2000
2575.ClAI
2510.SphI
2079.MluI

FIG. 21

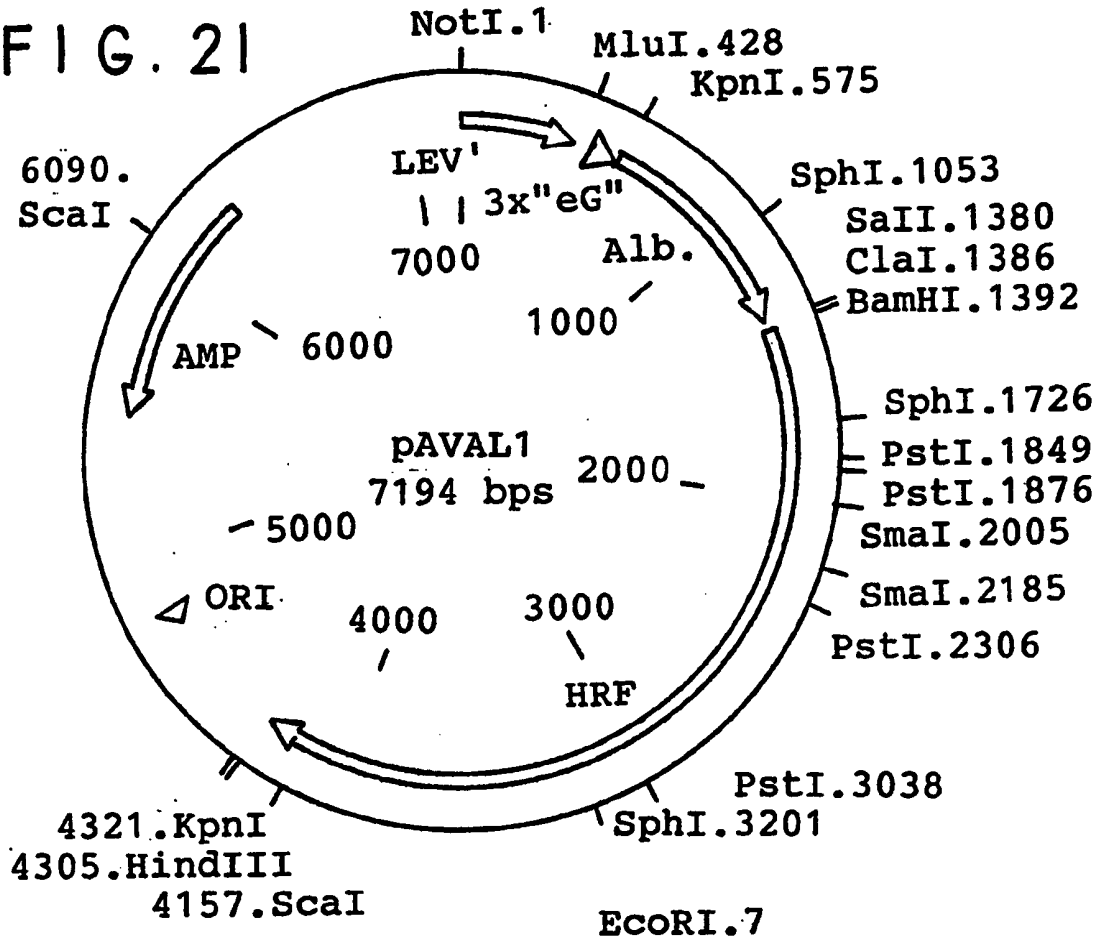

NotI.1
MluI.428
KpnI.575
SphI.1053
SalII.1380
ClaI.1386
BamHI.1392
SphI.1726
PstI.1849
PstI.1876
SmaI.2005
SmaI.2185
PstI.2306
PstI.3038
SphI.3201
4321.KpnI
4305.HindIII
4157.ScaI
6090.
ScaI
LEV
3x"eG"
7000
Alb.
1000
AMP
6000
pAVAL1
7194 bps
2000
5000
ORI
4000
3000
HRF

FIG.22

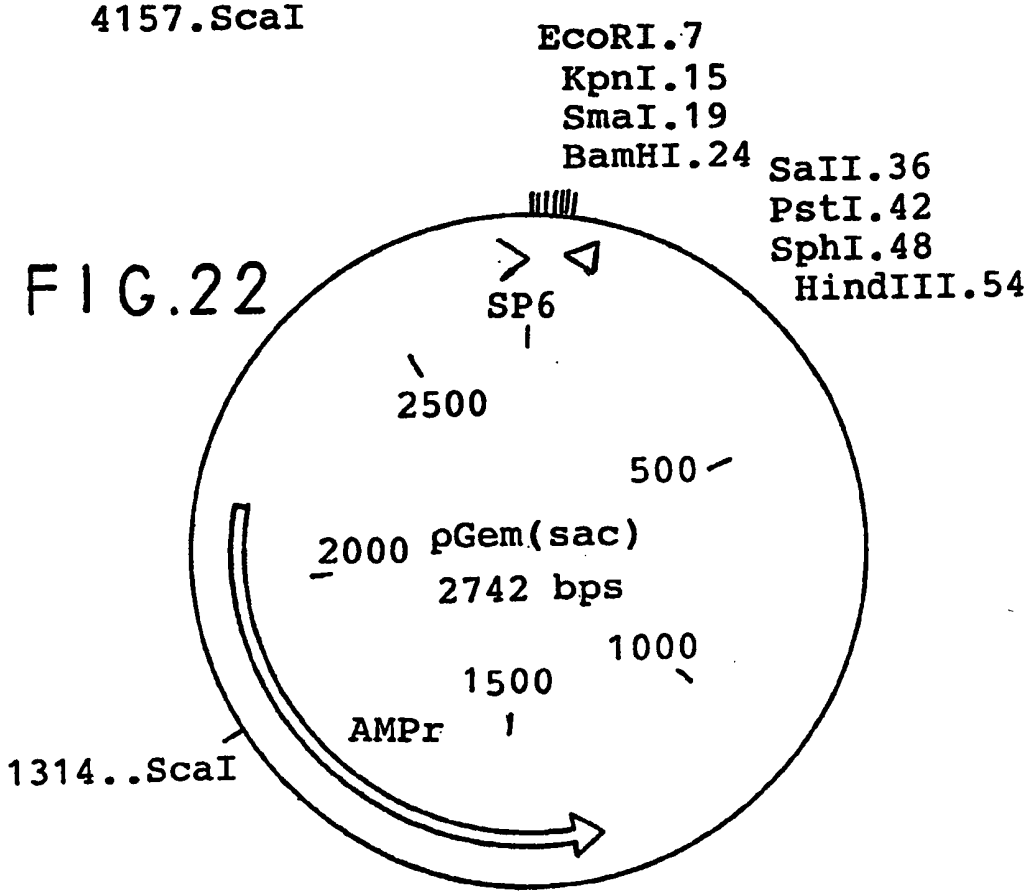

EcoRI.7
KpnI.15
SmaI.19
BamHI.24
SalII.36
PstI.42
SphI.48
HindIII.54
SP6
2500
500
2000
pGem(sac)
2742 bps
1000
1500
AMPr
1314..ScaI

FIG. 24

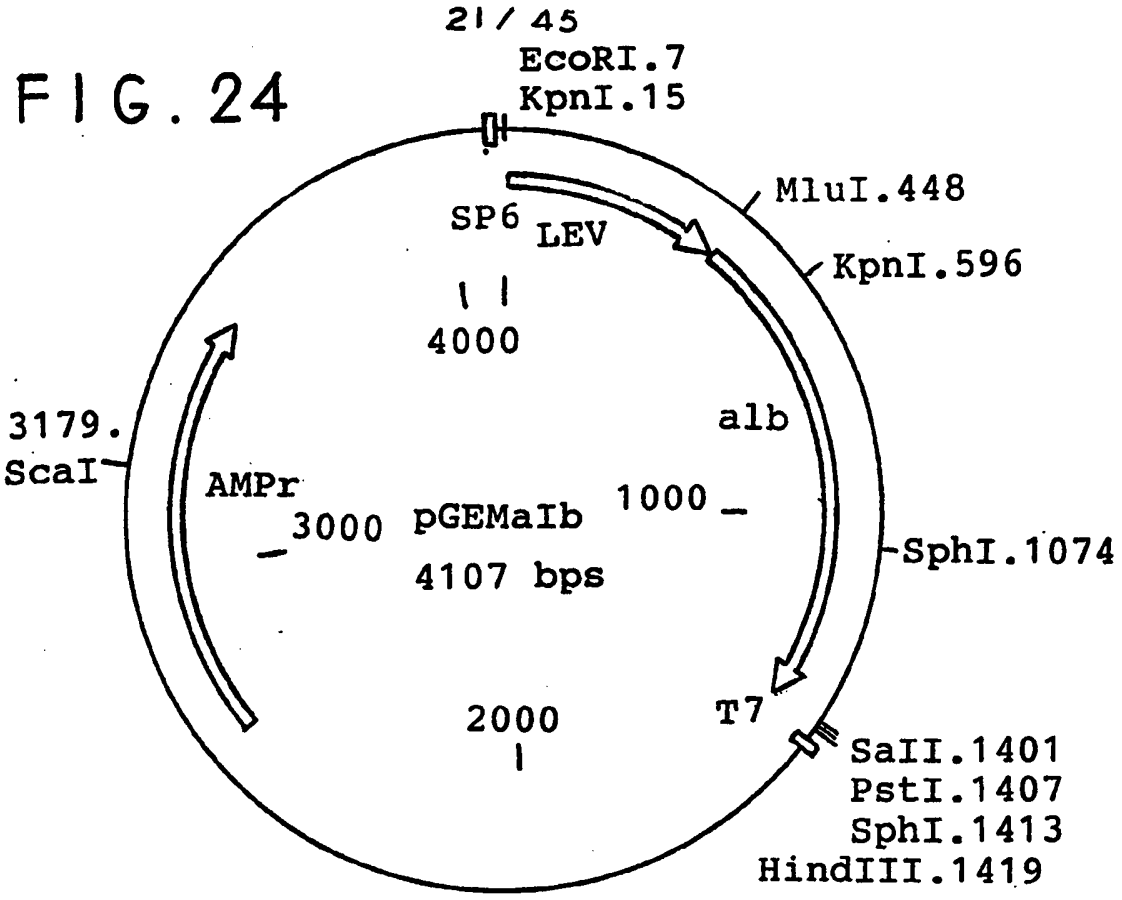

EcoRI.7
KpnI.15
MluI.448
KpnI.596
SP6
LEV
4000
alb
3179.
ScaI
AMPr
3000
pGEMaIb
1000
4107 bps
SphI.1074
2000
T7
SaII.1401
PstI.1407
SphI.1413
HindIII.1419

FIG. 25

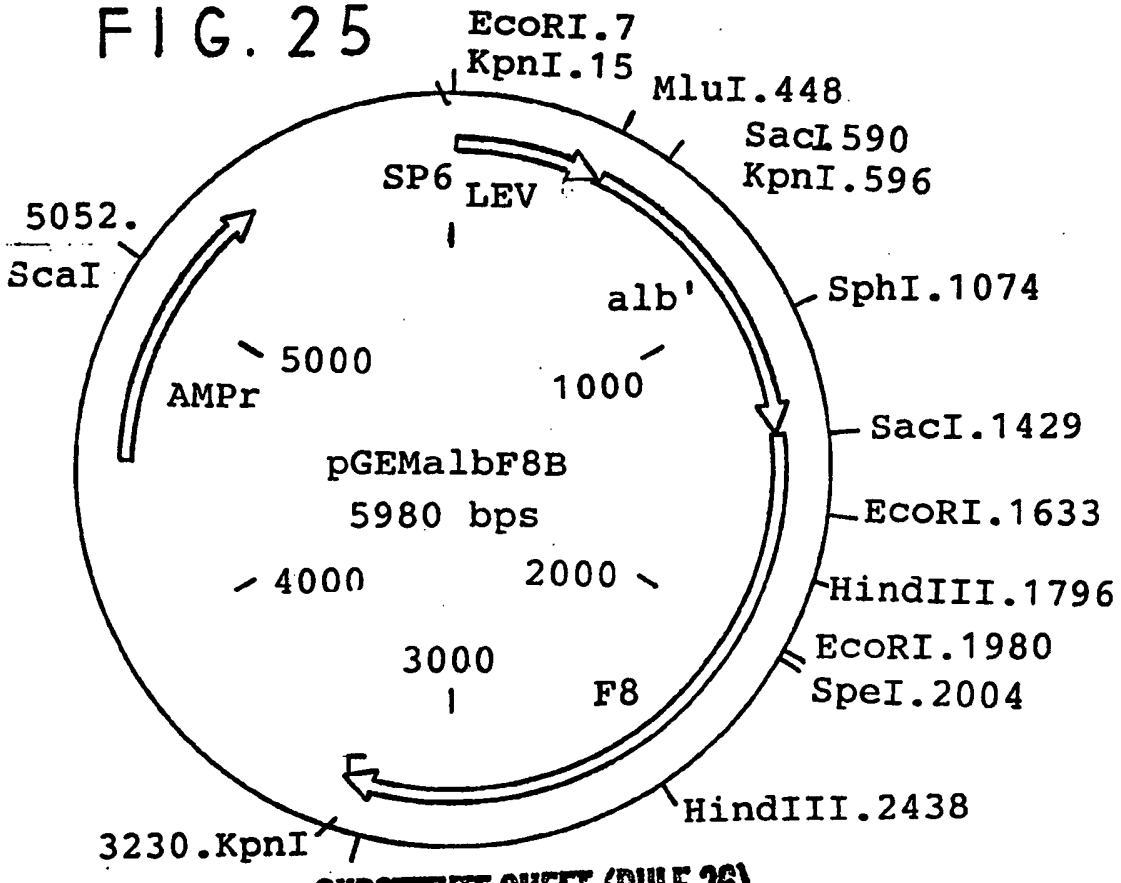

EcoRI.7
KpnI.15
MluI.448
SacI.590
KpnI.596
SP6
LEV
5052.
ScaI
alb'
SphI.1074
5000
AMPr
1000
SacI.1429
pGEMalbF8B
5980 bps
EcoRI.1633
4000
2000
HindIII.1796
EcoRI.1980
3000
F8
SpeI.2004
HindIII.2438
3230.KpnI

FIG. 26

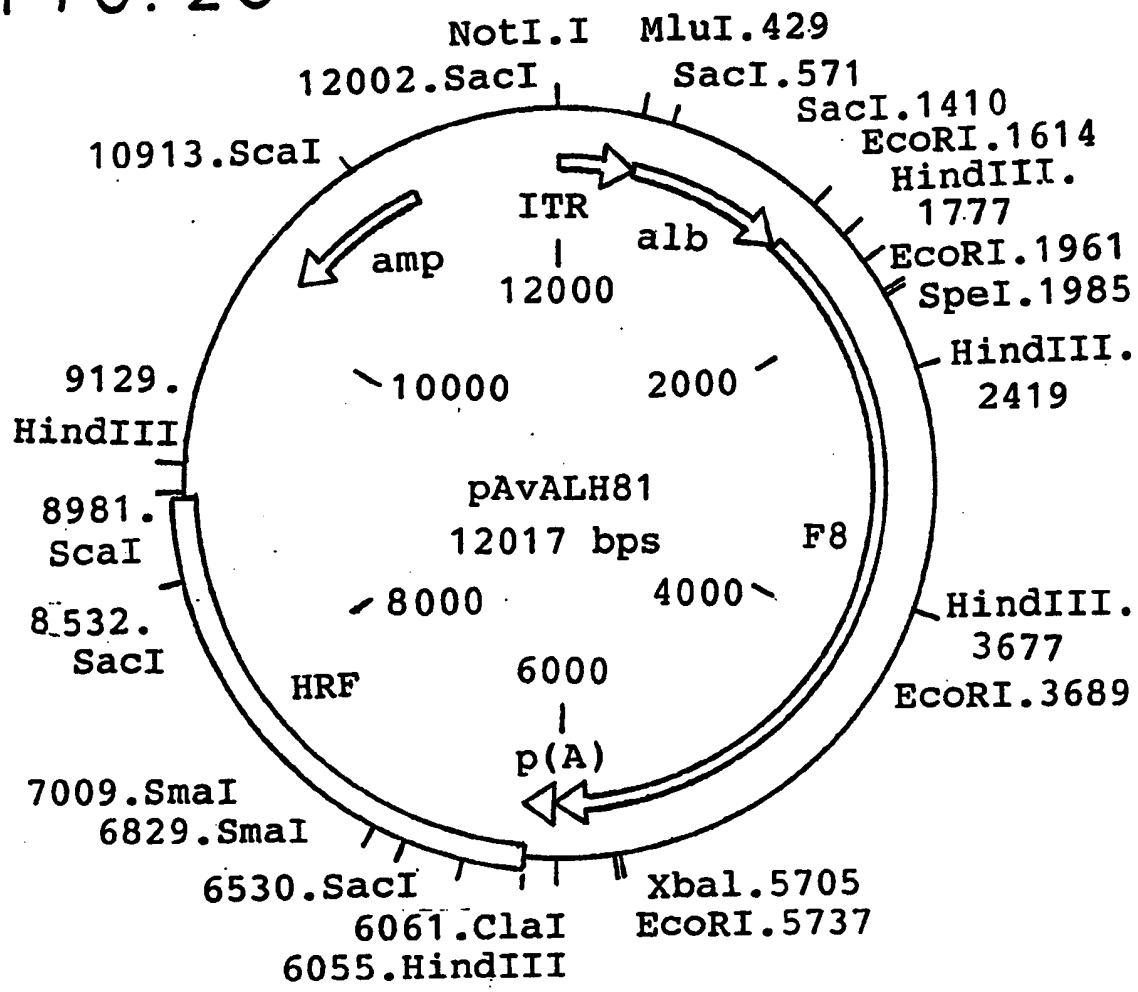

NotI.I
MluI.429
12002.SacI
SacI.571
SacI.1410
EcoRI.1614
HindIII.
1777
10913.ScaI
ITR
alb
amp
12000
EcoRI.1961
SpeI.1985
HindIII.
2419
9129.
HindIII
10000
2000
pAvALH81
12017 bps
F8
8981.
ScaI
8000
4000
8532.
SacI
HindIII.
3677
HRF
6000
EcoRI.3689
p(A)
7009.SmaI
6829.SmaI
6530.SacI
6061.ClaI
6055.HindIII
Xbal.5705
EcoRI.5737

FIG. 27

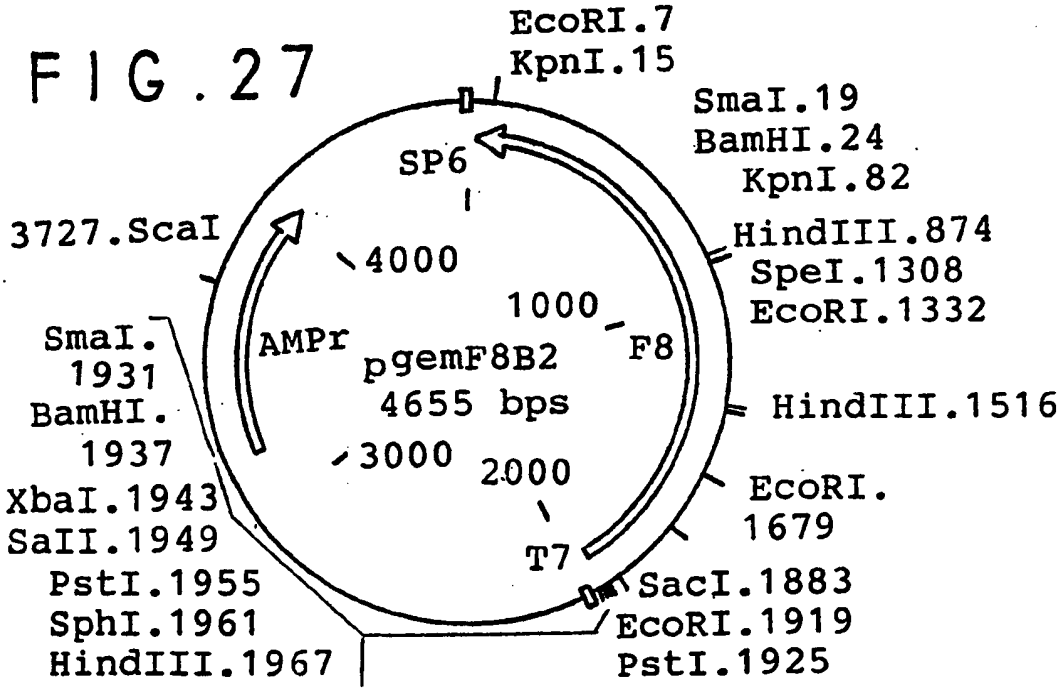
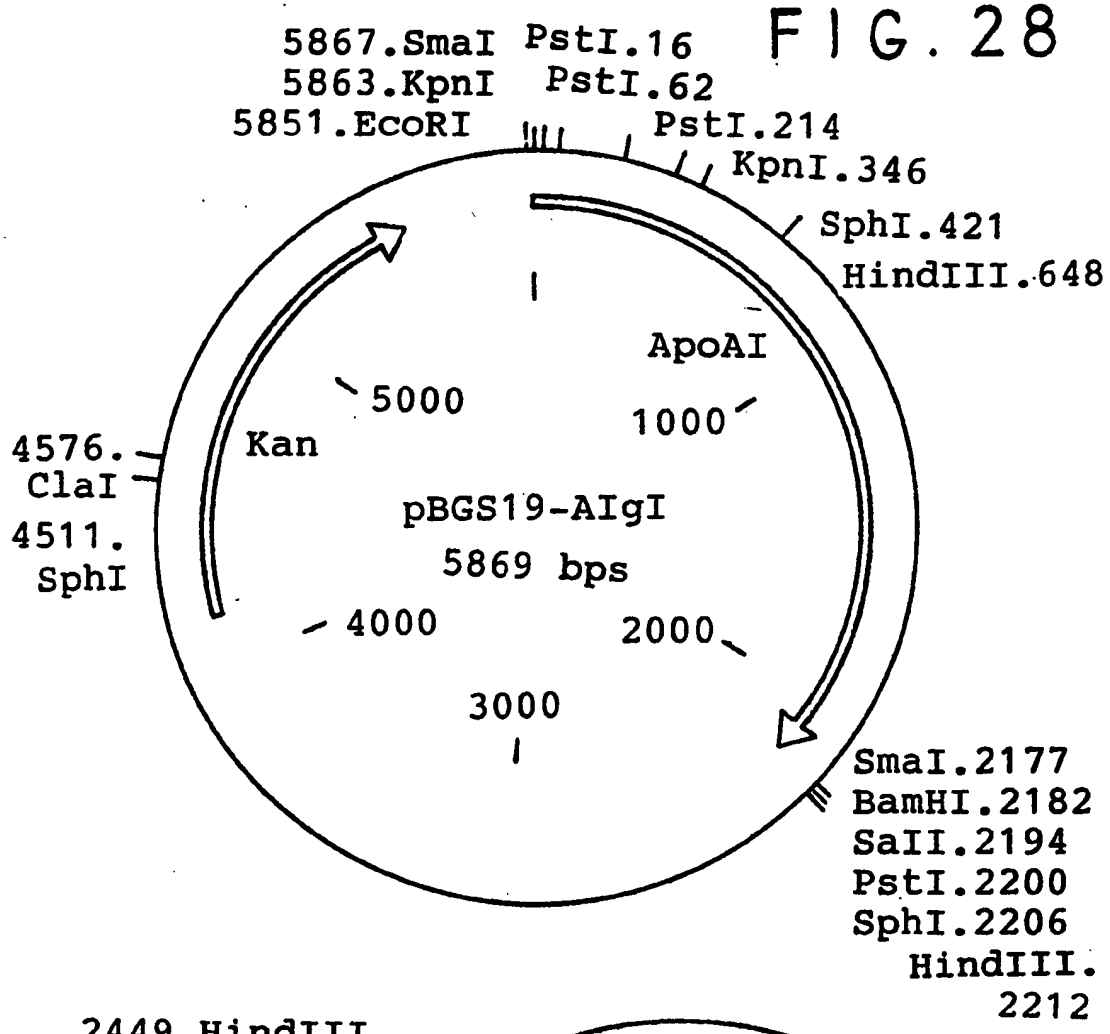

EcoRI.7
KpnI.15
SmaI.19
BamHI.24
KpnI.82
SP6
3727.ScaI
HindIII.874
SpeI.1308
EcoRI.1332
4000
1000
SmaI.
1931
AMPr
pgemF8B2
F8
4655 bps
HindIII.1516
BamHI.
1937
3000
2000
XbaI.1943
EcoRI.
1679
SaII.1949
T7
PstI.1955
SacI.1883
SphI.1961
EcoRI.1919
HindIII.1967
PstI.1925

FIG. 28


5867.SmaI
5863.KpnI
5851.EcoRI
PstI.16
PstI.62
PstI.214
KpnI.346
SphI.421
HindIII.648
ApoAI
1000
5000
Kan
4576.
ClaI
4511.
SphI
pBGS19-AIgI
5869 bps
4000
2000
3000
SmaI.2177
BamHI.2182
SaII.2194
PstI.2200
SphI.2206
HindIII.
2212

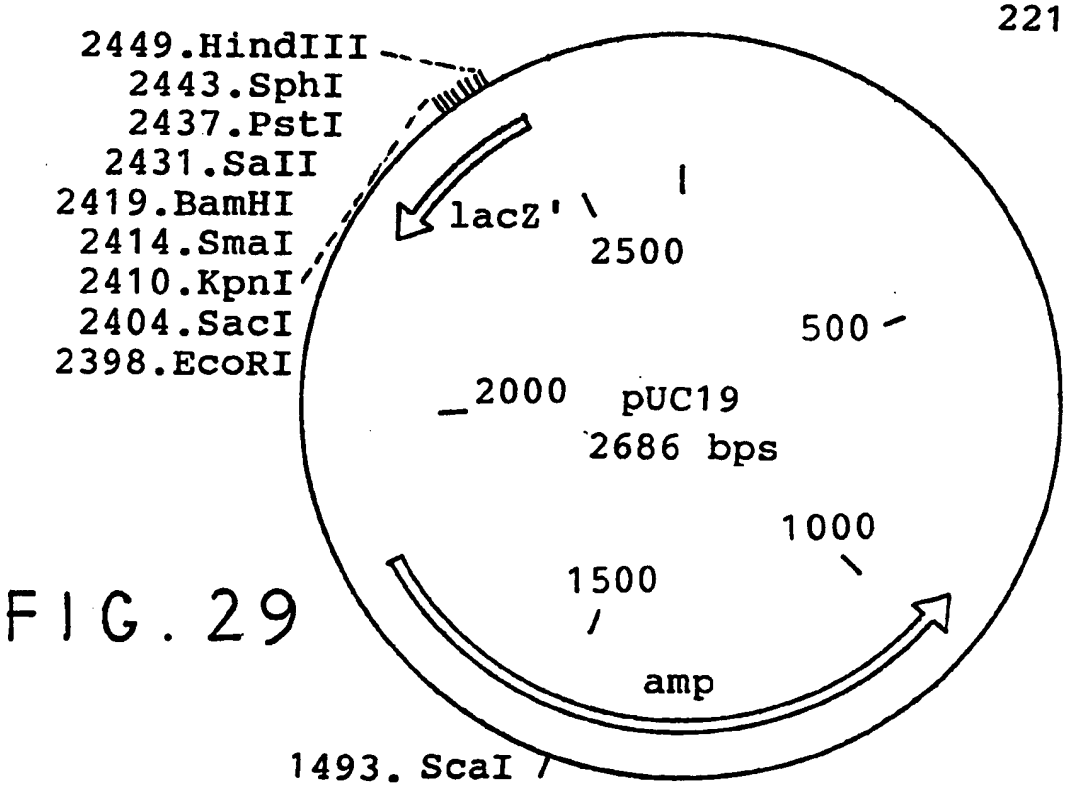

2449.HindIII
2443.SphI
2437.PstI
2431.SaII
2419.BamHI
2414.SmaI
2410.KpnI
2404.SacI
2398.EcoRI
lacZ'
2500
500
2000
pUC19
2686 bps
1000
1500
amp
1493. ScaI

FIG. 29

FIG. 30

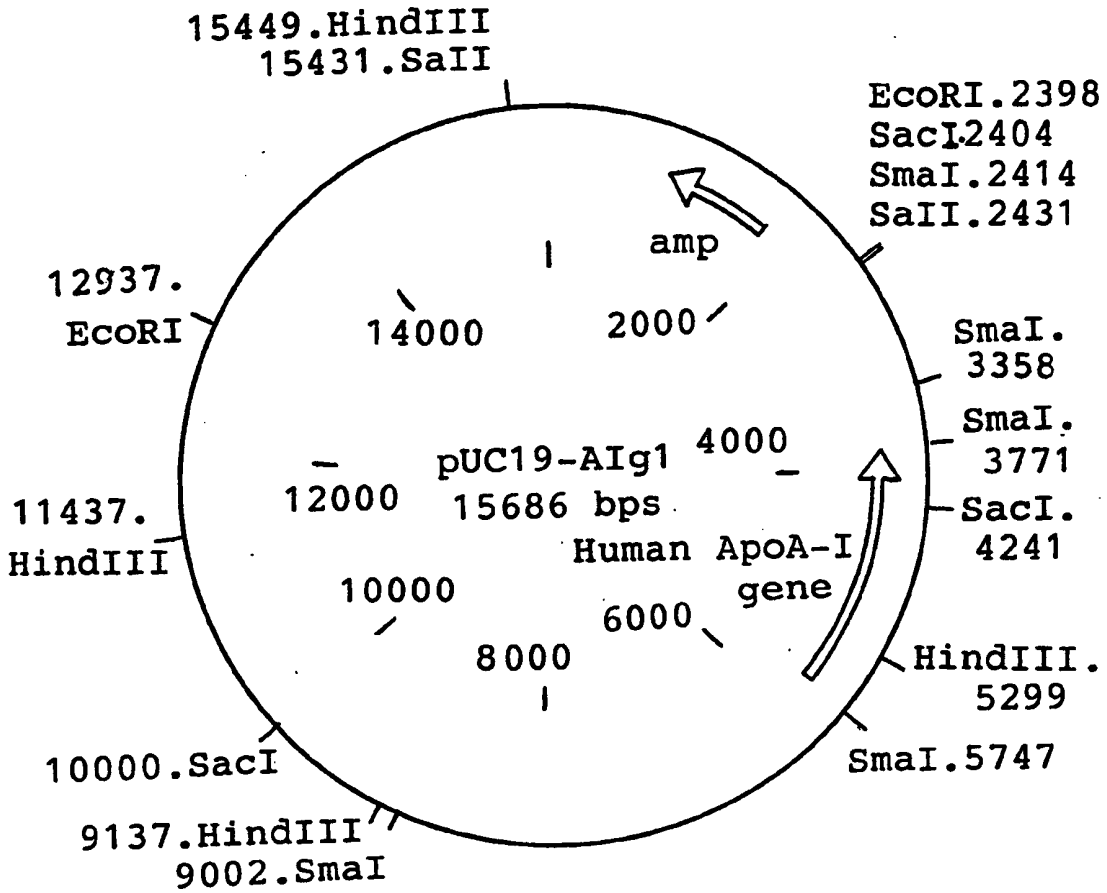

15449.HindIII
15431.SaII
EcoRI.2398
SacI.2404
SmaI.2414
SaII.2431
amp
12937.
EcoRI
14000
2000
SmaI.
3358
SmaI.
3771
4000
pUC19-AIg1
15686 bps
12000
11437.
HindIII
Human ApoA-I
gene
SacI.
4241
10000
6000
8000
HindIII.
5299
10000.SacI
SmaI.5747
9137.HindIII
9002.SmaI

FIG. 33

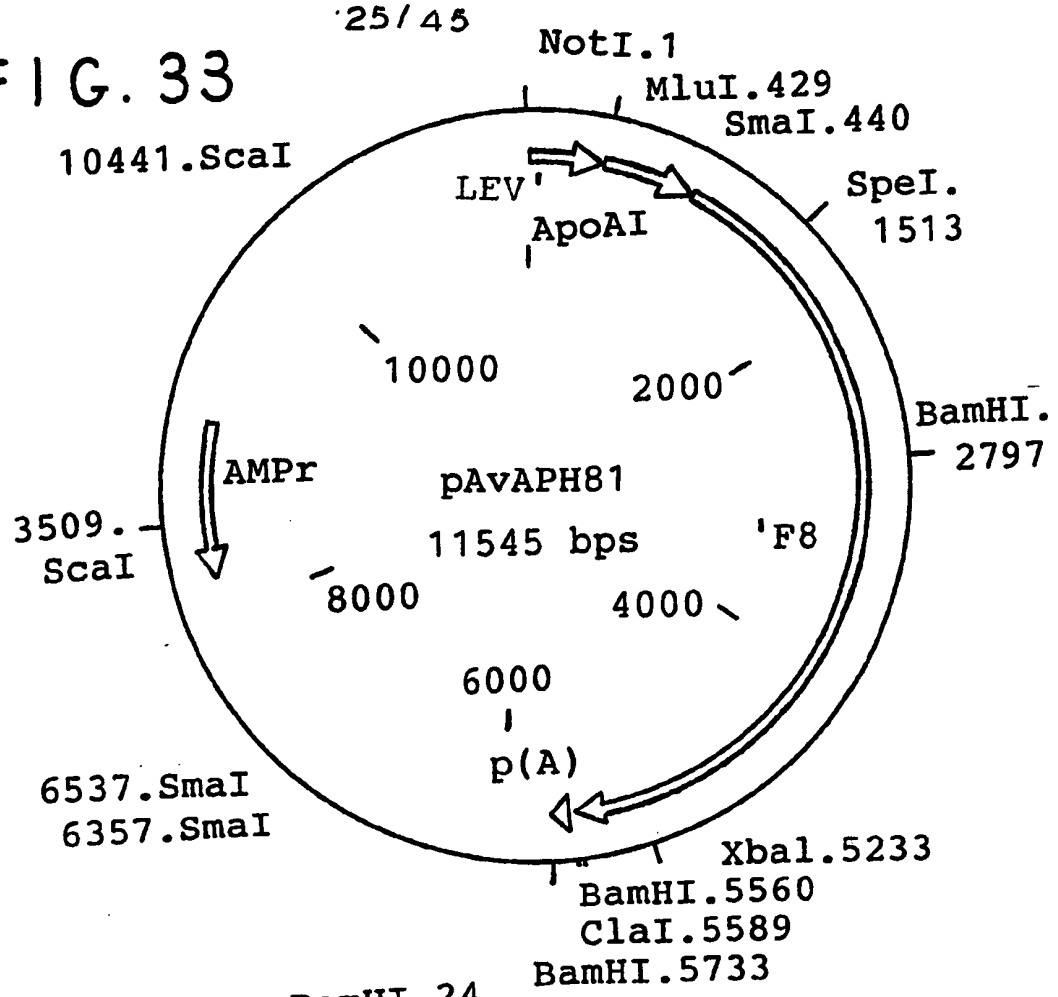

NotI.1
MluI.429
SmaI.440
10441.ScaI
LEV'
ApoAI
SpeI.
1513
10000
2000
BamHI.
2797
AMPr
pAvAPH81
11545 bps
'F8
3509.
ScaI
8000
4000
6000
p(A)
6537.SmaI
6357.SmaI
XbaI.5233
BamHI.5560
ClaI.5589
BamHI.5733

FIG. 32

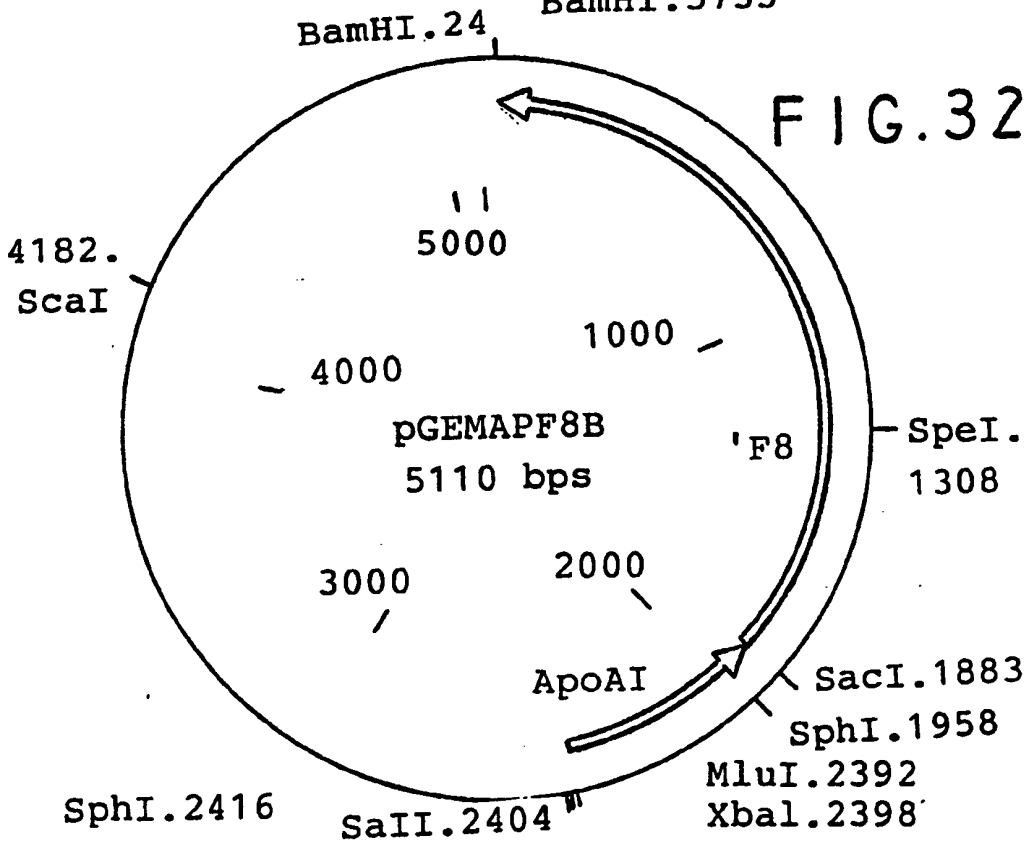

BamHI.24
5000
4182.
ScaI
1000
4000
pGEMAPF8B
5110 bps
'F8
SpeI.
1308
3000
2000
ApoAI
SacI.1883
SphI.1958
MluI.2392
XbaI.2398
SphI.2416
SaII.2404

FIG. 34

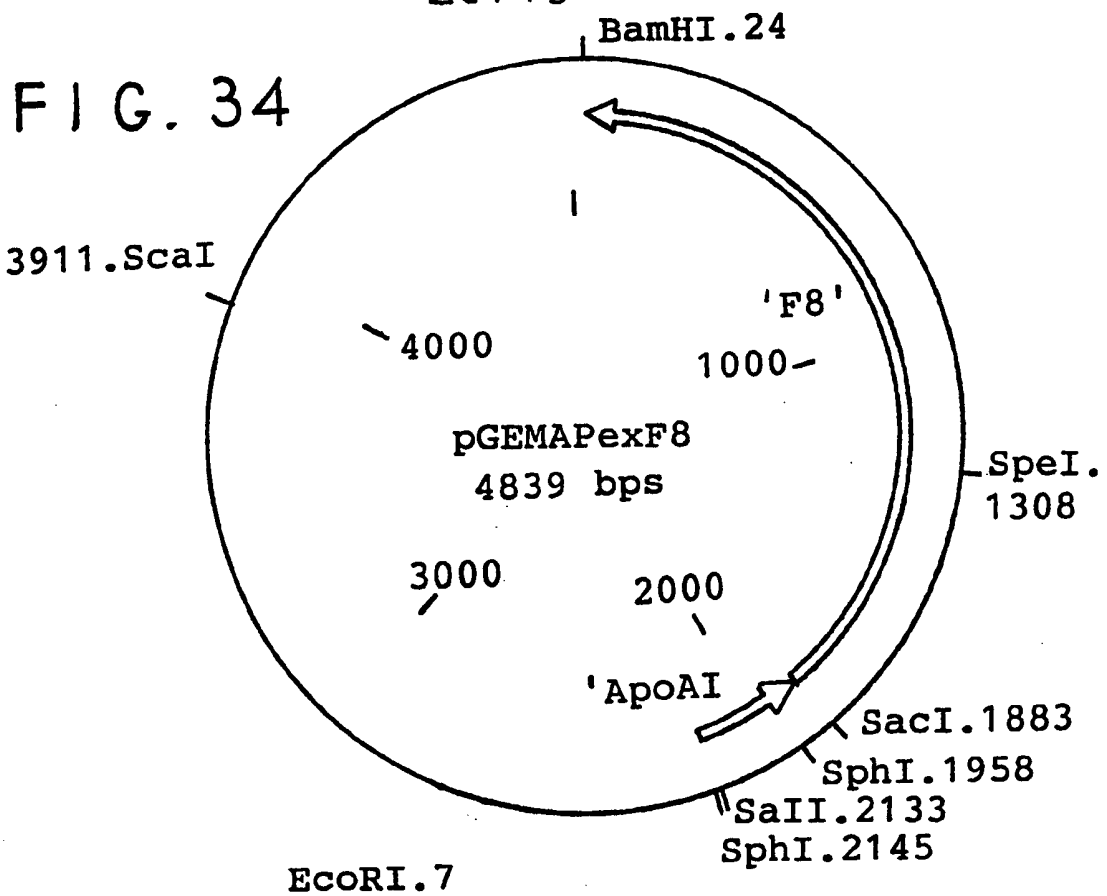

BamHI.24
3911.ScaI
'F8'
4000
1000
pGEMAPexF8
4839 bps
SpeI.
1308
3000
2000
'ApoAI
SacI.1883
SphI.1958
SaII.2133
SphI.2145

FIG. 35

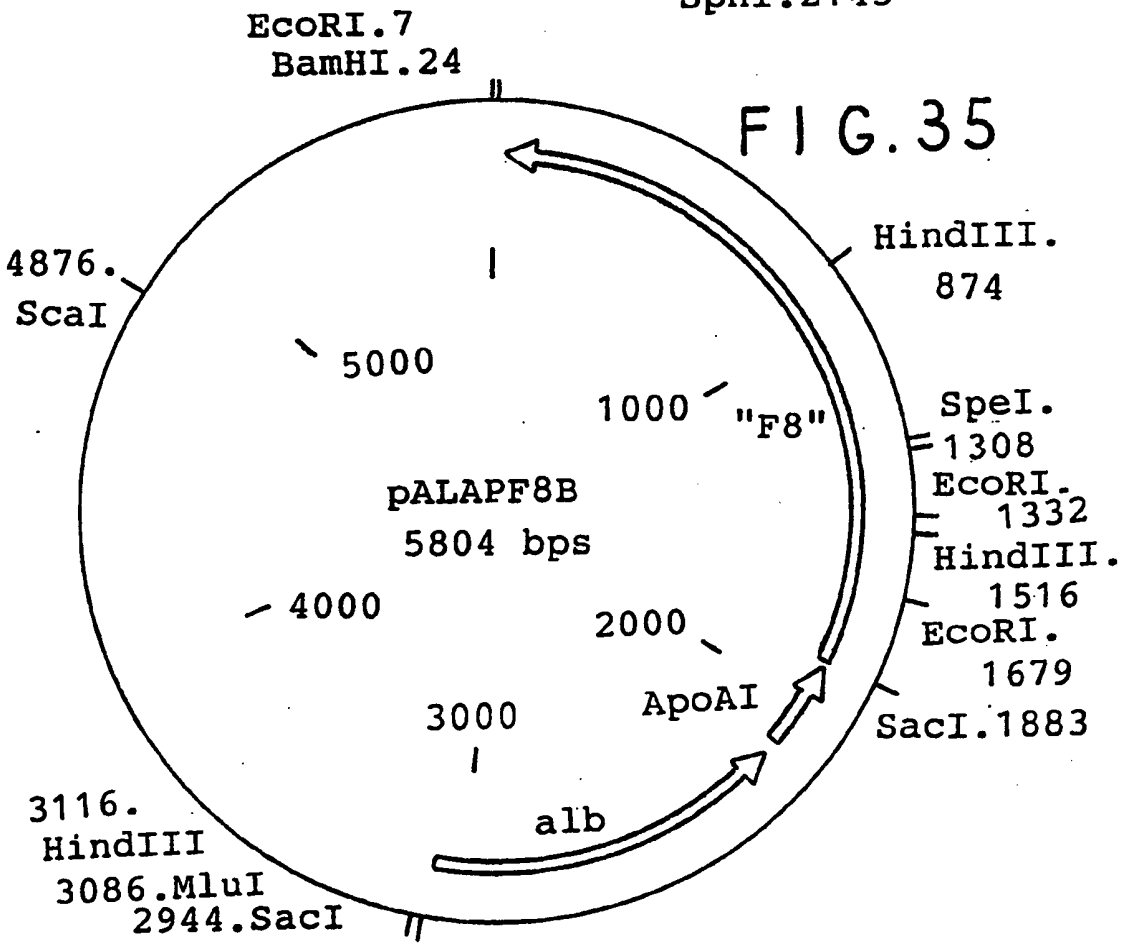

EcoRI.7
BamHI.24
4876.
ScaI
HindIII.
874
5000
1000
"F8"
SpeI.
1308
EcoRI.
1332
pALAPF8B
5804 bps
HindIII.
1516
4000
2000
EcoRI.
1679
ApoAI
3000
SacI.1883
alb
3116.
HindIII
3086.MluI
2944.SacI

FIG. 36

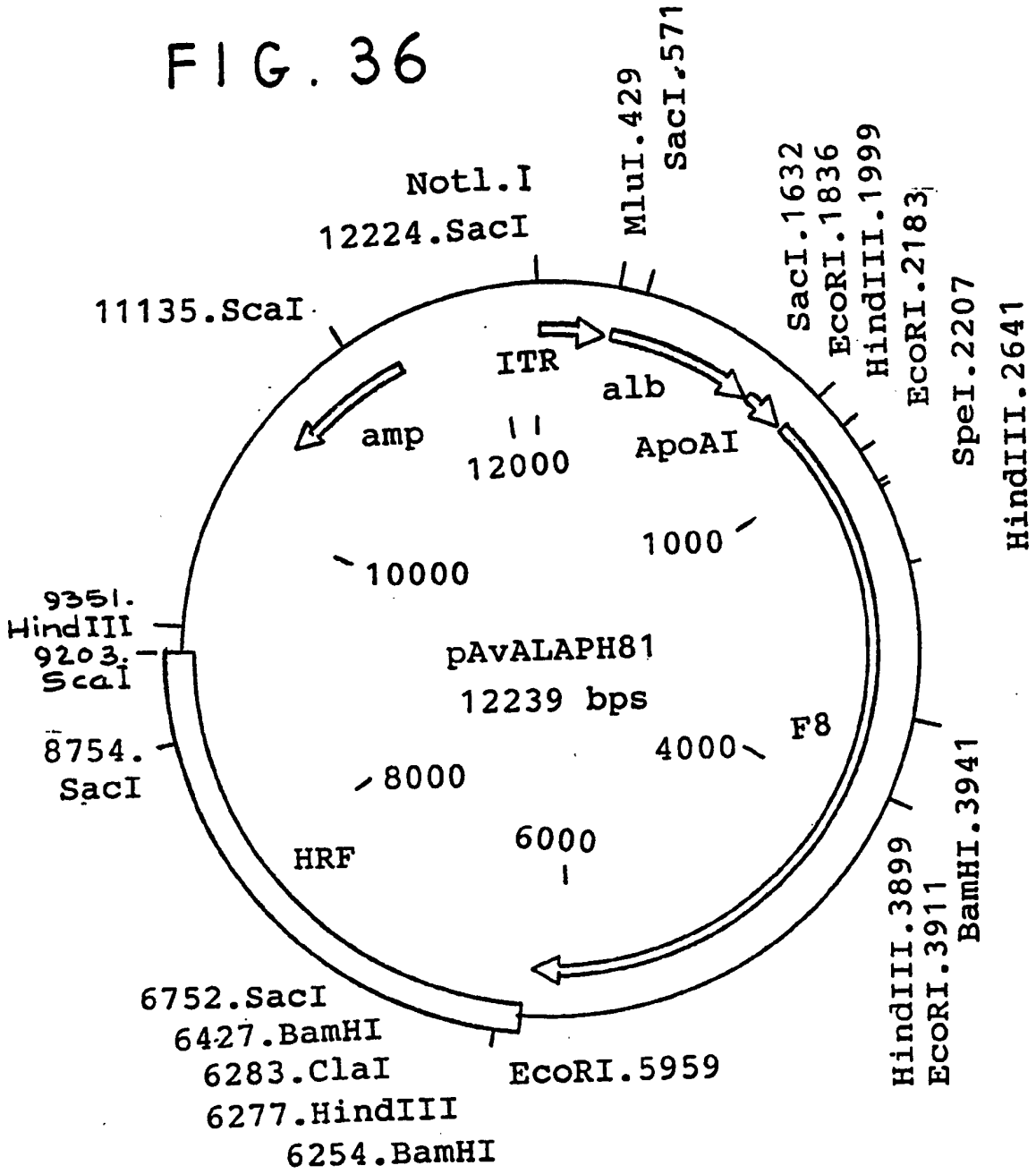

NotI.I
12224.SacI
MluI.429
SacI.571
11135.ScaI
SacI.1632
EcoRI.1836
HindIII.1999
EcoRI.2183
SpeI.2207
HindIII.2641
ITR
alb
amp
12000
ApoAI
1000
10000
9351.
HindIII
9203.
ScaI
pAvALAPH81
12239 bps
F8
4000
8754.
SacI
8000
6000
HRF
HindIII.3899
EcoRI.3911
BamHI.3941
6752.SacI
6427.BamHI
6283.ClaI
6277.HindIII
6254.BamHI
EcoRI.5959

FIG. 37

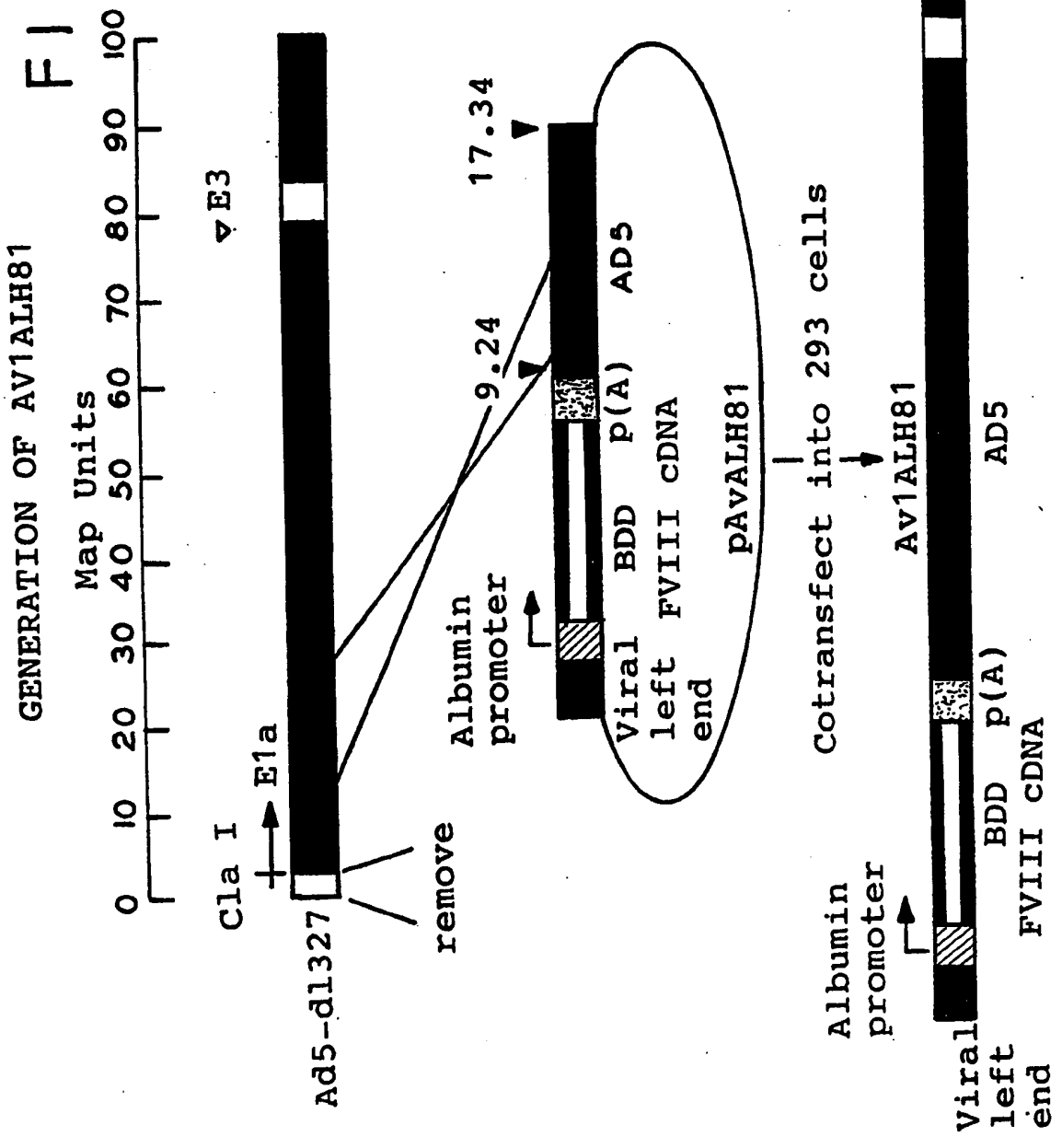

GENERATION OF AV1ALH81
Map Units
0
10
20
30
40
50
60
70
80
90
100
E3
17.34
9.24
Cla I
E1a
Ad5-d1327
remove
Albumin promoter
Viral left end
BDD FVIII cDNA
p(A)
AD5
pAVALH81
Cotransfect into 293 cells
Av1ALH81
Albumin promoter
Viral left end
BDD FVIII cDNA
p(A)
AD5

FIG. 38

RESTRICTION DIGESTION ANALYSIS

OF Av1ALH81 DNA

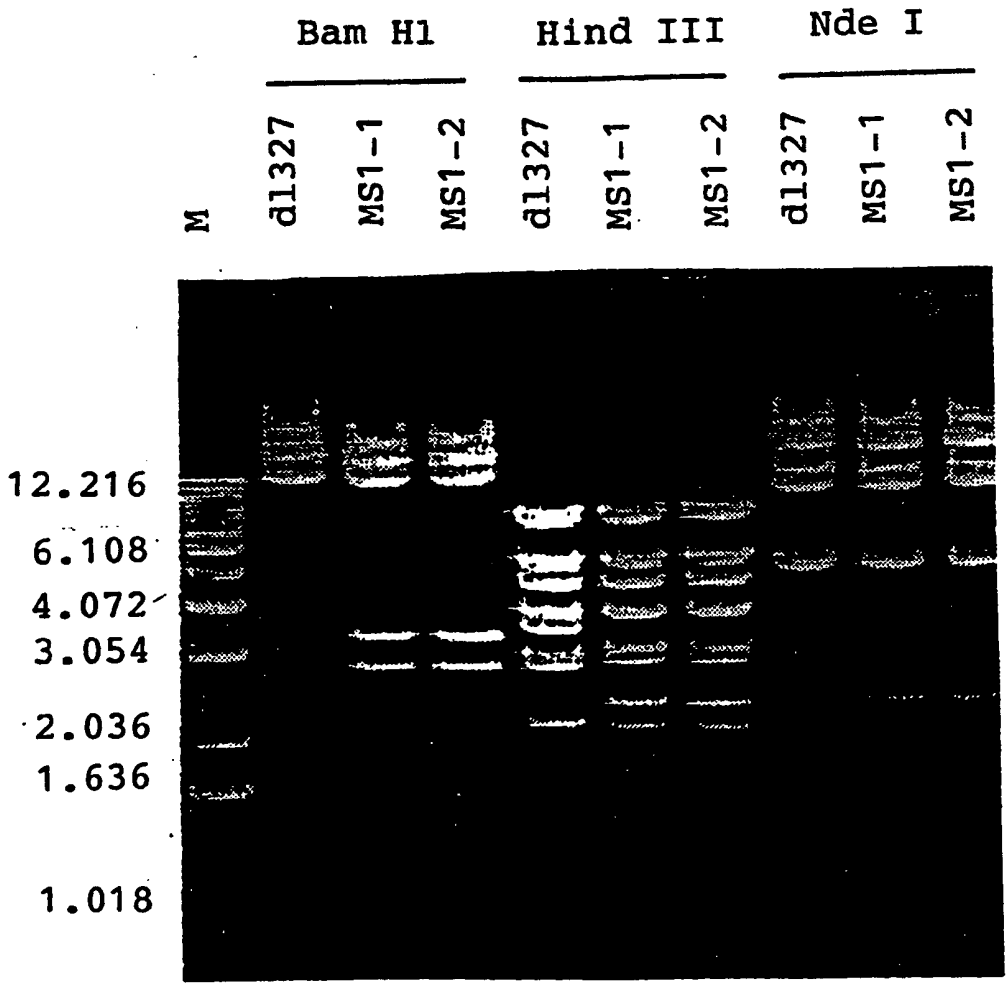


FIG. 39

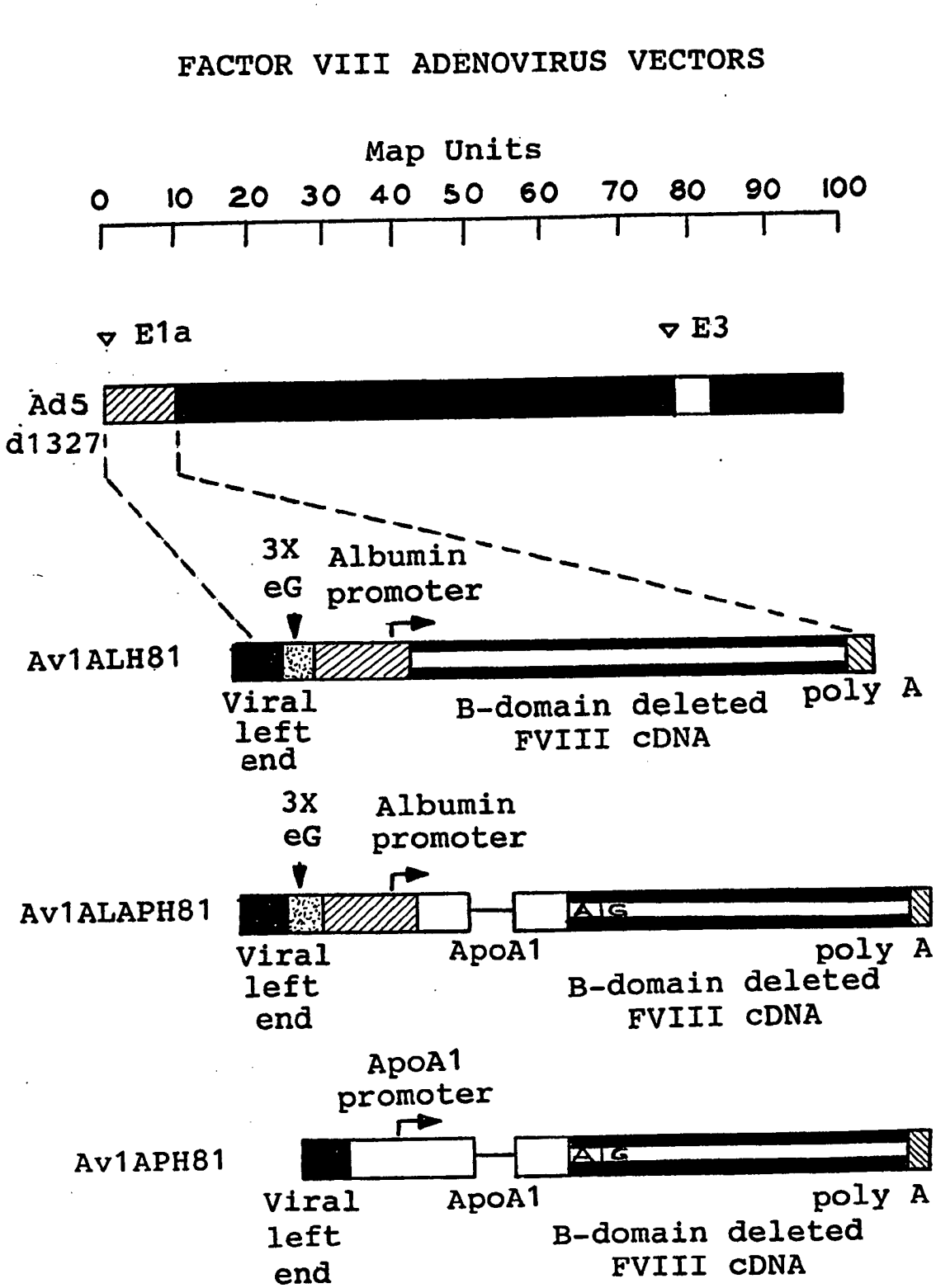
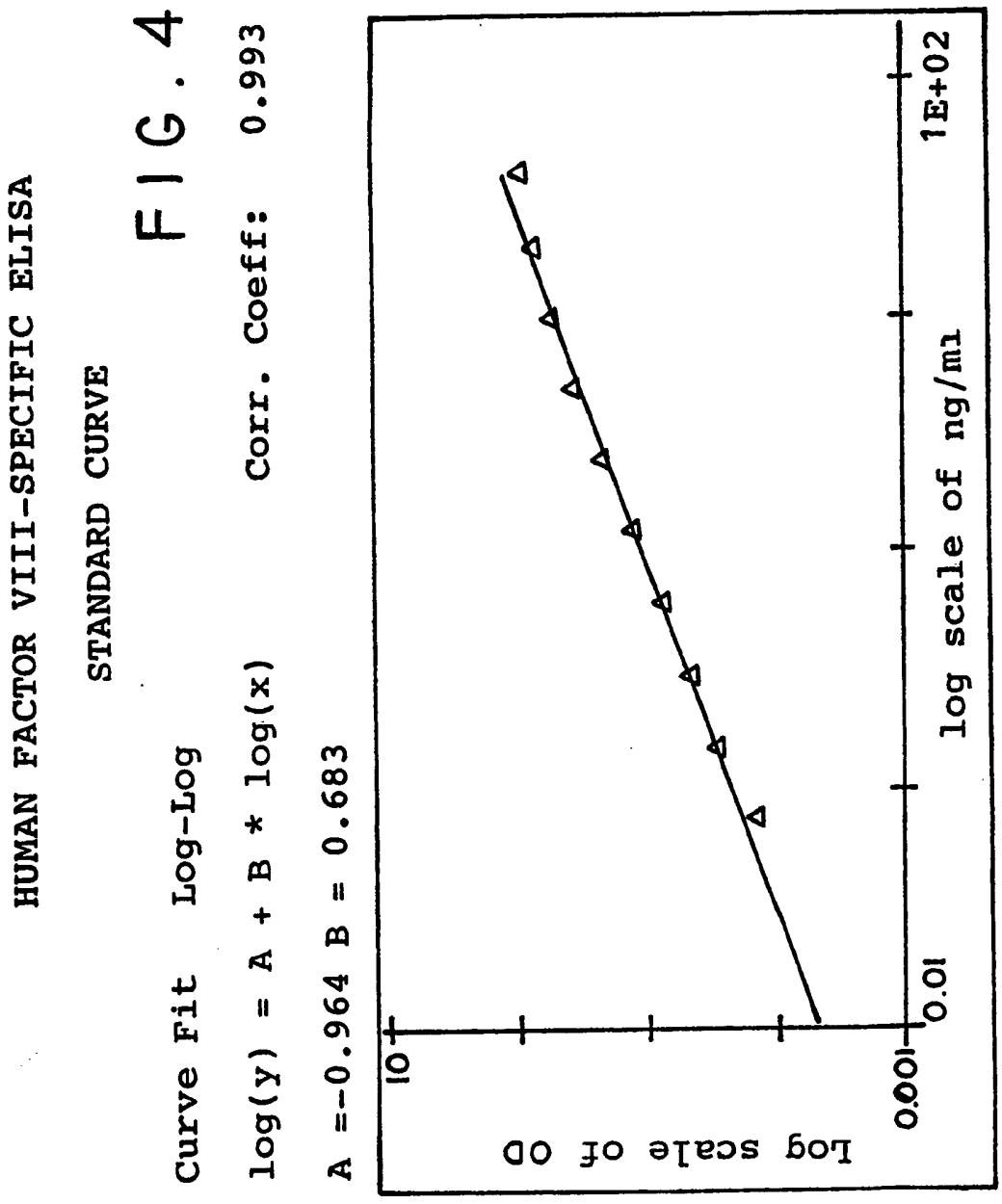

FACTOR VIII ADENOVIRUS VECTORS
Map Units
0 10 20 30 40 50 60 70 80 90 100
E1a
E3
Ad5
d1327
3X eG
Albumin promoter
Av1ALH81
Viral left end
B-domain deleted FVIII cDNA
poly A
3X eG
Albumin promoter
Av1ALAPH81
Viral left end
ApoA1
AIG
B-domain deleted FVIII cDNA
poly A
ApoA1 promoter
Av1APH81
Viral left end
ApoA1
AIG
B-domain deleted FVIII cDNA
poly A

FIG. 40


HUMAN FACTOR VIII-SPECIFIC ELISA
STANDARD CURVE
Curve Fit Log-Log
log(y) = A + B * log(x)
A =-0.964 B = 0.683
Corr. Coeff: 0.993
Log scale of OD
10
0.001
0.01
1E+02
log scale of ng/ml

HALF-LIFE OF HUMAN B-DOMAIN DELETED FACTOR VIII IN MOUSE PLASMA

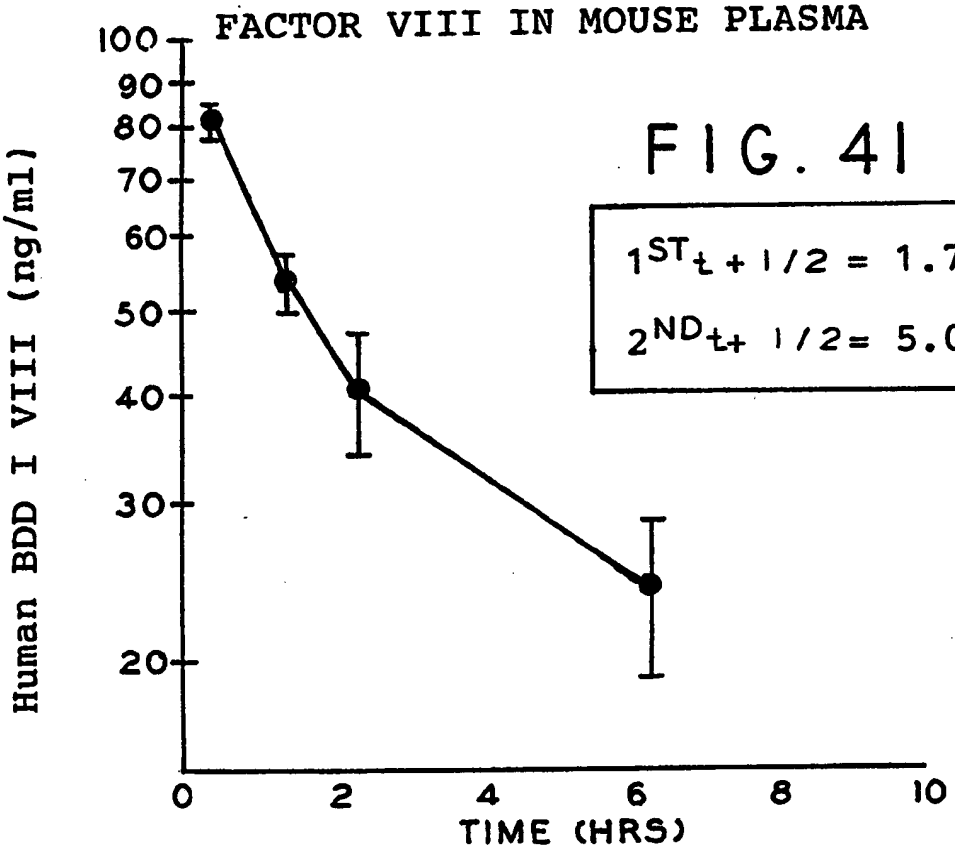


FIG. 41

$1^{ST}t + 1/2 = 1.7$ hr

$2^{ND}t + 1/2 = 5.0$ hr

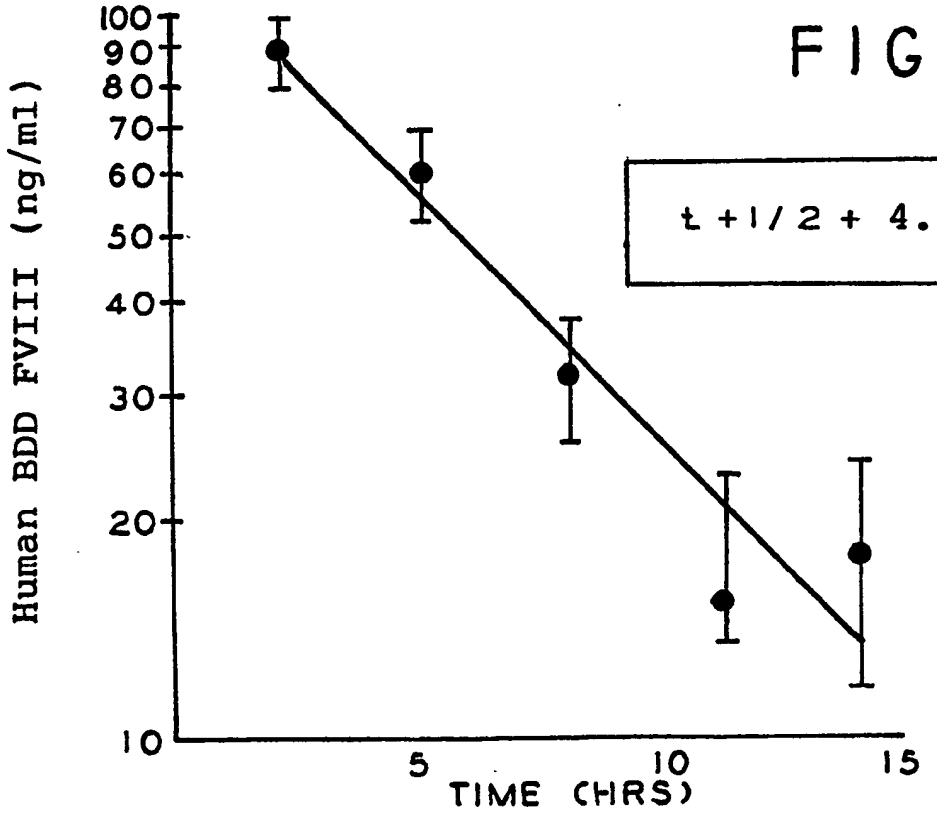


FIG. 42

t + 1/2 + 4.4 hr

IN VIVO EXPRESSION OF FACTOR VIII

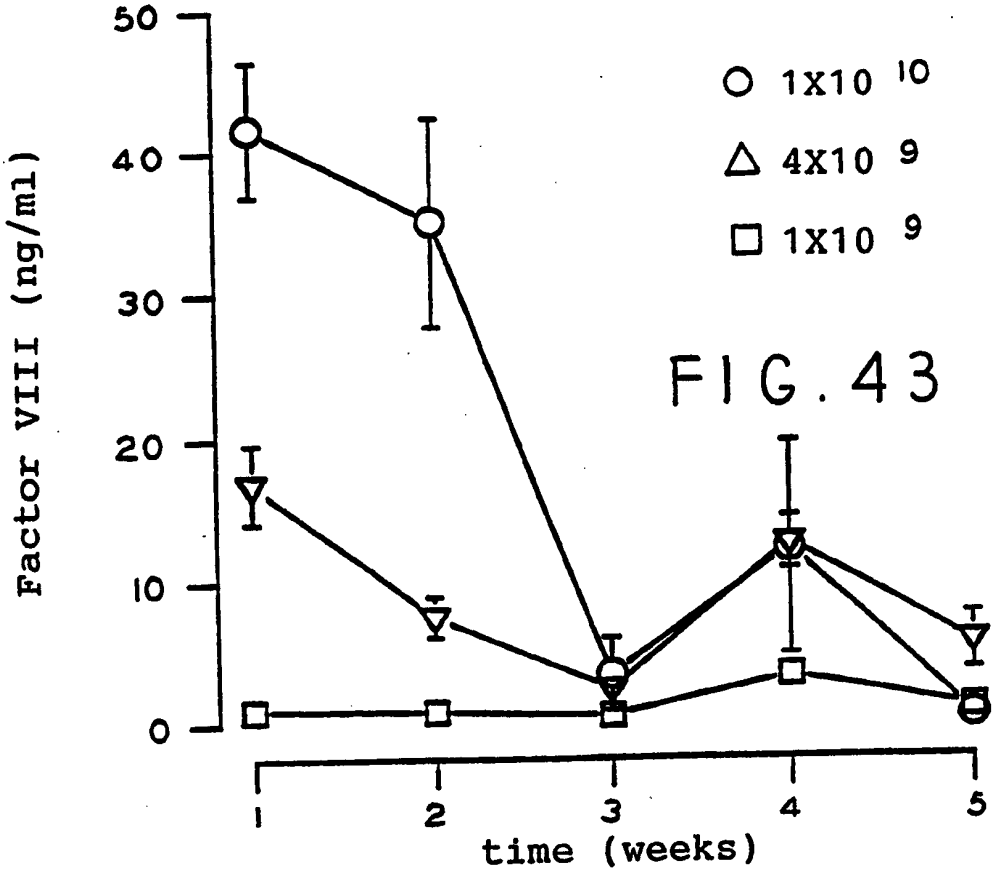


FIG. 43

HUMAN FACTOR VIII
HALF-LIFE IN MICE

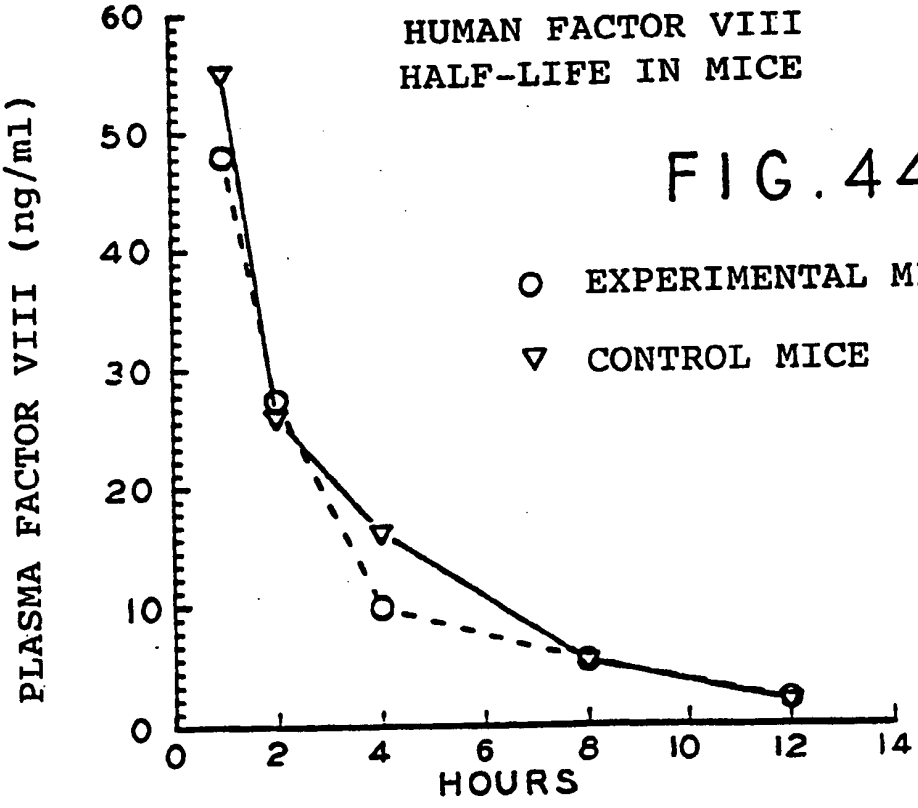


FIG. 44

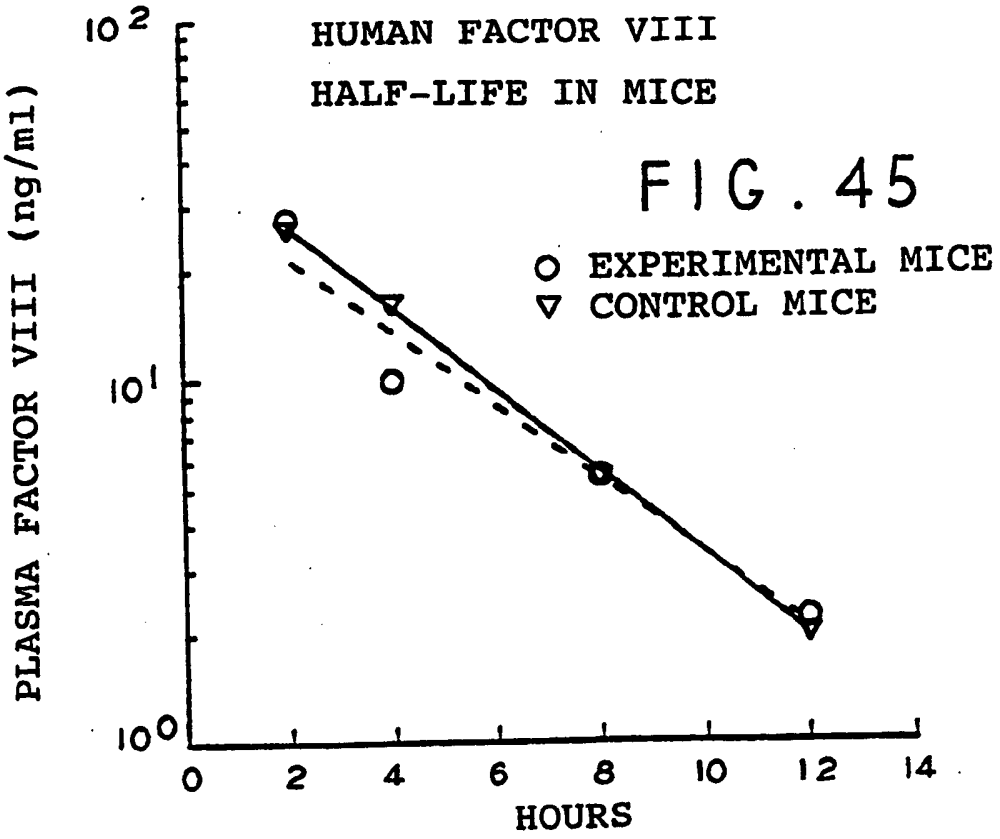

HUMAN FACTOR VIII
HALF-LIFE IN MICE
PLASMA FACTOR VIII (ng/ml)
10^2
10^1
10^0
0 2 4 6 8 10 12 14
HOURS
EXPERIMENTAL MICE
CONTROL MICE

FIG. 45

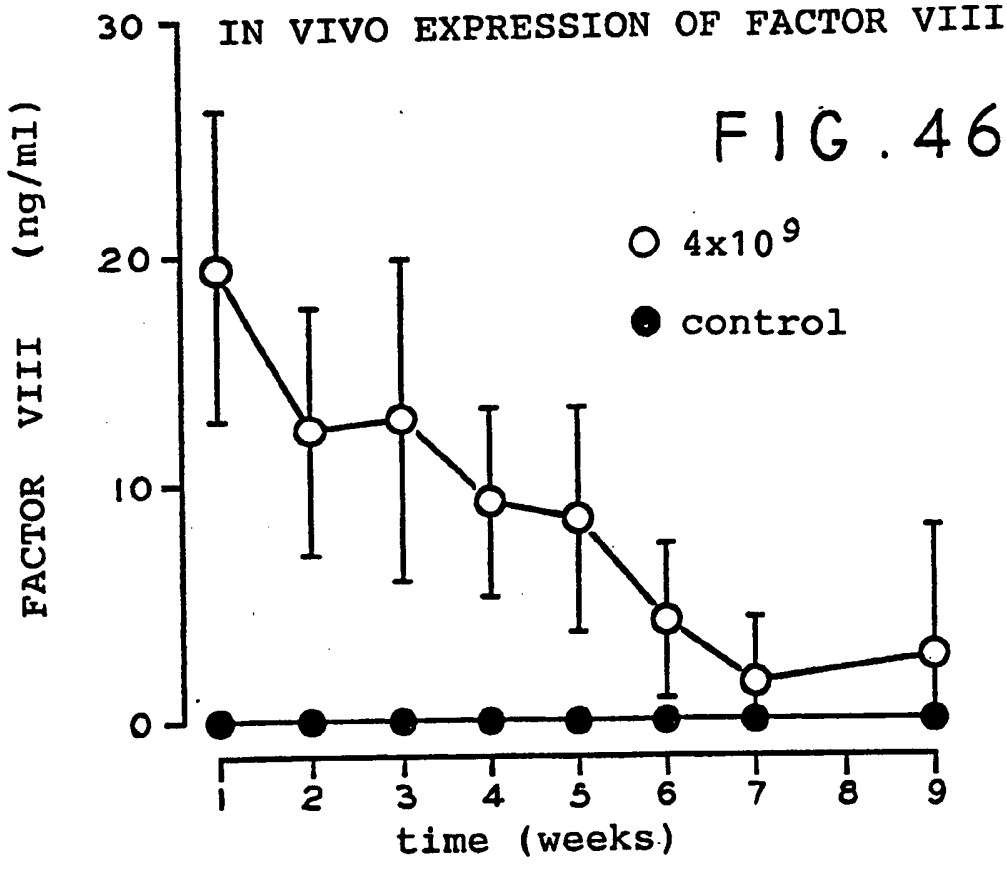
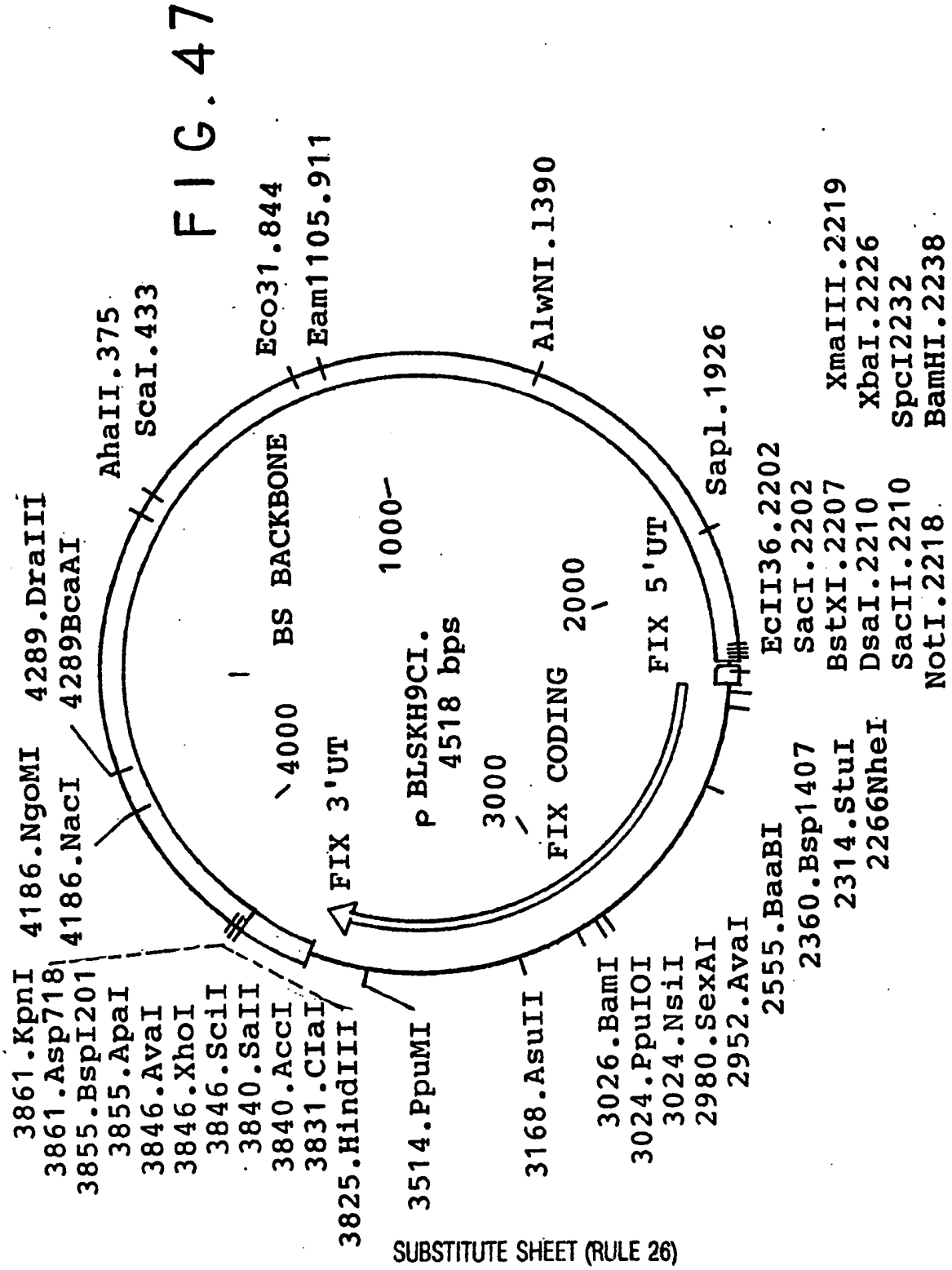

IN VIVO EXPRESSION OF FACTOR VIII
FACTOR VIII (ng/ml)
30
20
10
0
1 2 3 4 5 6 7 8 9
time (weeks)
$4x10^9$
control

FIG. 46

FIG. 47


p BLSKH9CI.
4518 bps
1000
2000
3000
4000
BS BACKBONE
FIX 3'UT
FIX CODING
FIX 5'UT
3861.KpnI
3861.Asp718
3855.BspI201
3855.ApaI
3846.AvaI
3846.XhoI
3846.SciI
3840.SaII
3840.AccI
3831.ClaI
3825.HindIII
4186.NgoMI
4186.NacI
4289.DraIII
4289BcaAI
AhaII.375
ScaI.433
Eco31.844
Eam1105.911
AlwNI.1390
Sap1.1926
EcII36.2202
SacI.2202
BstXI.2207
DsaI.2210
SacII.2210
NotI.2218
XmaIII.2219
XbaI.2226
SpcI2232
BamHI.2238
2555.BaaBI
2360.Bsp1407
2314.StuI
2266NheI
3514.PpuMI
3168.AsuII
3026.BamI
3024.PpuIOI
3024.NsiI
2980.SexAI
2952.AvaI

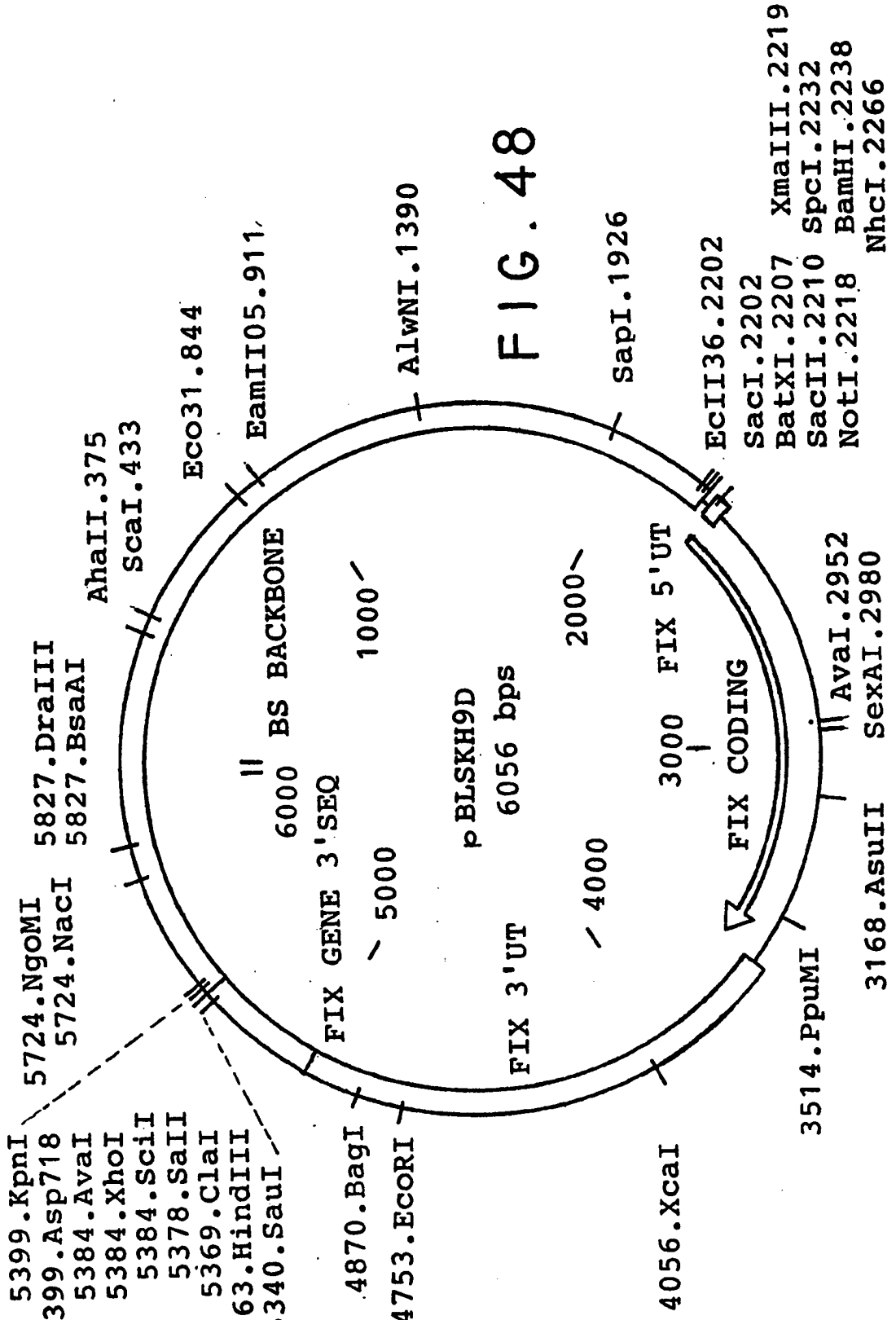

pBLSKH9D
6056 bps
1000
2000
3000
4000
5000
6000
BS BACKBONE
FIX GENE 3'SEQ
FIX 3'UT
FIX CODING
FIX 5'UT
5399.KpnI
5399.Asp718
5384.AvaI
5384.XhoI
5384.SciI
5378.SalI
5369.ClaI
5363.HindIII
5340.SauI
5724.NgoMI
5724.NaeI
5827.DraIII
5827.BsaAI
AhaII.375
ScaI.433
Eco31.844
EamII05.911
AlwNI.1390
SapI.1926
EcII36.2202
SacI.2202
BatXI.2207
SacII.2210
NotI.2218
XmaIII.2219
SpcI.2232
BamHI.2238
NheI.2266
AvaI.2952
SexAI.2980
3168.AsuII
3514.PpuMI
4056.XcaI
4753.EcoRI
4870.BagI

FIG. 48

FIG. 49

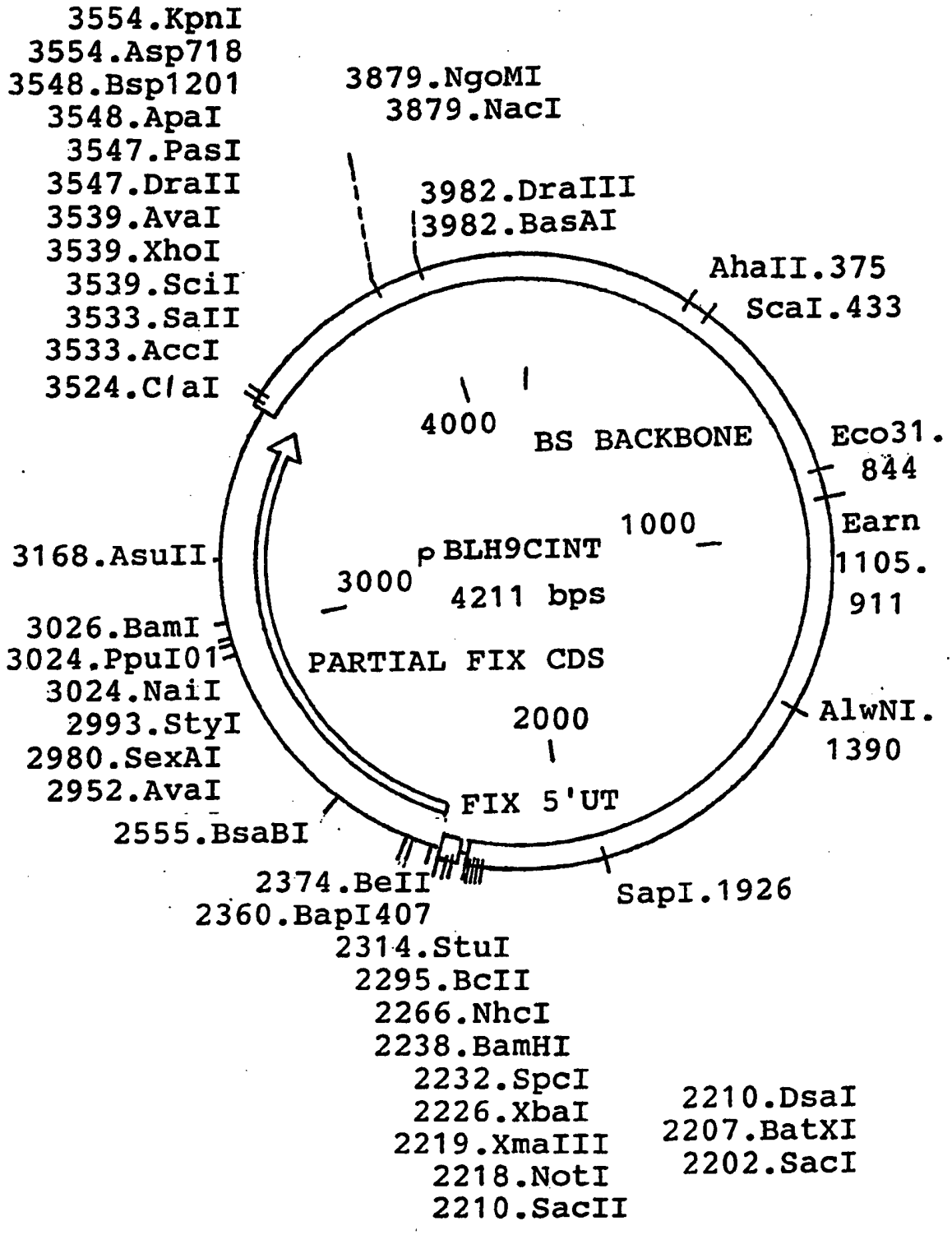

3554.KpnI
3554.Asp718
3548.Bsp1201
3548.ApaI
3547.PasI
3547.DraII
3539.AvaI
3539.XhoI
3539.SciI
3533.SaII
3533.AccI
3524.ClaI
3879.NgoMI
3879.NacI
3982.DraIII
3982.BasAI
AhaII.375
ScaI.433
Eco31.
844
Earn
1105.
911
AlwNI.
1390
SapI.1926
2210.DsaI
2207.BatXI
2202.SacI
2218.NotI
2210.SacII
2232.SpcI
2226.XbaI
2219.XmaIII
2238.BamHI
2266.NhcI
2295.BcII
2314.StuI
2360.BapI407
2374.BeII
2555.BsaBI
2952.AvaI
2980.SexAI
2993.StyI
3024.NaiI
3024.PpuI01
3026.BamI
3168.AsuII
4000
BS BACKBONE
1000
pBLH9CINT
3000
4211 bps
PARTIAL FIX CDS
2000
FIX 5'UT

FIG. 50

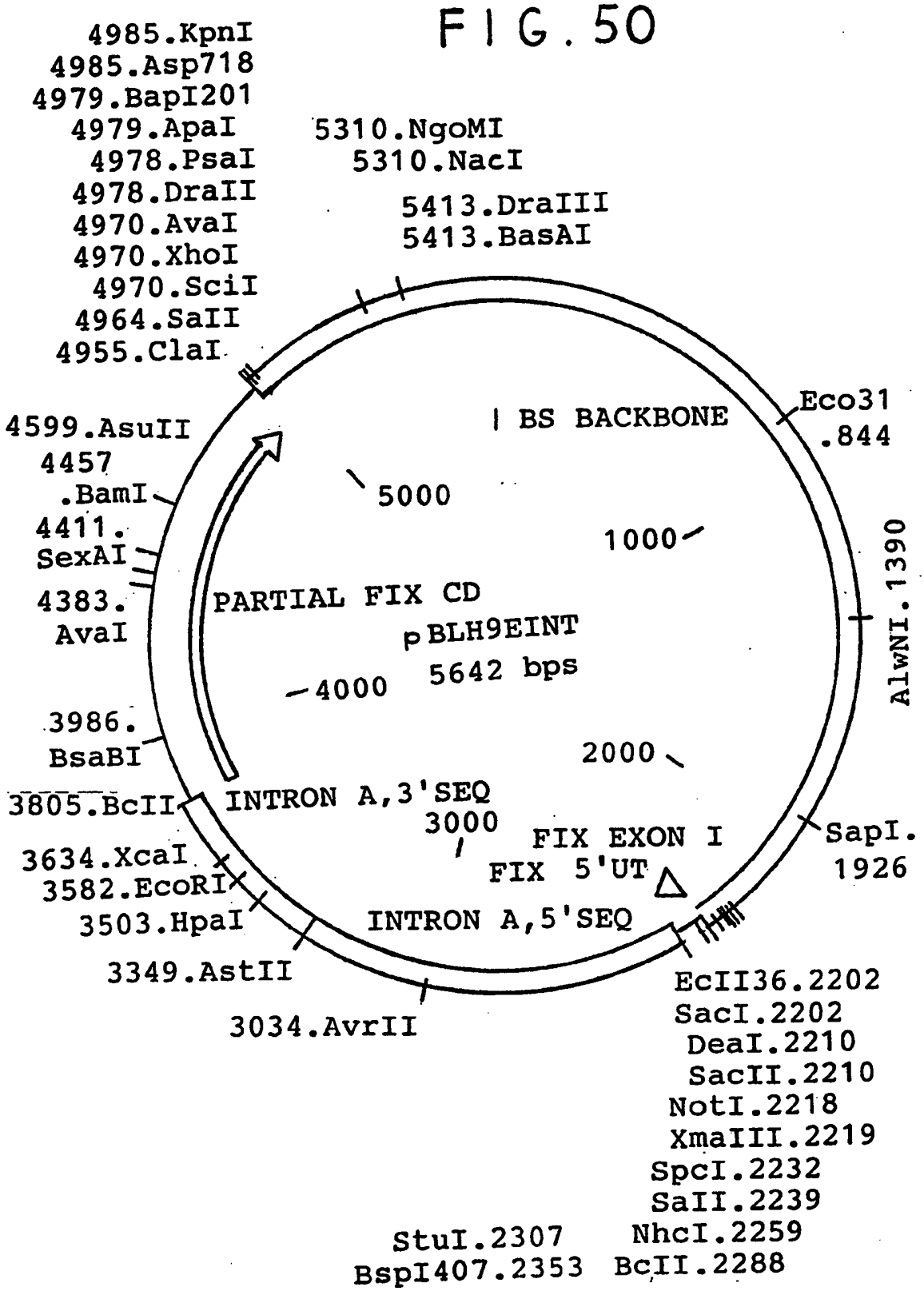

4985.KpnI
4985.Asp718
4979.BapI201
4979.ApaI
4978.PsaI
4978.DraII
4970.AvaI
4970.XhoI
4970.SciI
4964.SaII
4955.ClaI
4599.AsuII
4457
.BamI
4411.
SexAI
4383.
AvaI
3986.
BsaBI
3805.BclI
3634.XcaI
3582.EcoRI
3503.HpaI
3349.AstII
3034.AvrII
5310.NgoMI
5310.NacI
5413.DraIII
5413.BasAI
Eco31
.844
AlwNI.1390
SapI.
1926
EcII36.2202
SacI.2202
DeaI.2210
SacII.2210
NotI.2218
XmaIII.2219
SpcI.2232
SaII.2239
NhcI.2259
BcII.2288
StuI.2307
BspI407.2353
1 BS BACKBONE
5000
1000
2000
3000
4000
PARTIAL FIX CD
pBLH9EINT
5642 bps
INTRON A,3'SEQ
FIX EXON I
FIX 5'UT
INTRON A,5'SEQ

FIG. 51

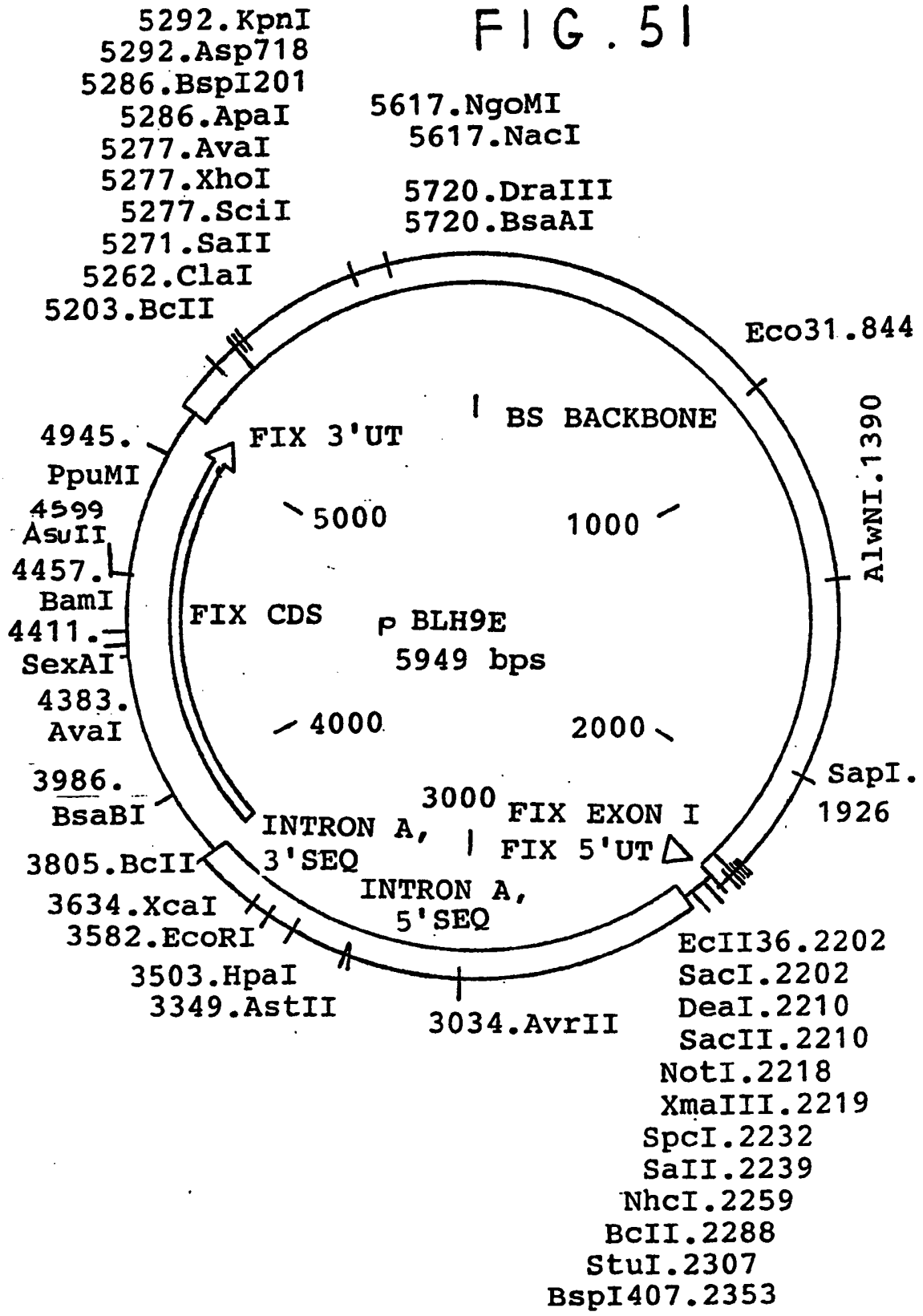

5292.KpnI
5292.Asp718
5286.BspI201
5286.ApaI
5277.AvaI
5277.XhoI
5277.SciI
5271.SaII
5262.ClaI
5203.BcII
5617.NgoMI
5617.NacI
5720.DraIII
5720.BsaAI
Eco31.844
AlwNI.1390
BS BACKBONE
FIX 3'UT
4945.
PpuMI
4599
AsuII
4457.
BamI
4411.
SexAI
4383.
AvaI
3986.
BsaBI
3805.BcII
3634.XcaI
3582.EcoRI
3503.HpaI
3349.AstII
5000
1000
2000
3000
4000
FIX CDS
p BLH9E
5949 bps
INTRON A,
3'SEQ
INTRON A,
5'SEQ
FIX EXON I
FIX 5'UT
SapI.
1926
3034.AvrII
EcII36.2202
SacI.2202
DeaI.2210
SacII.2210
NotI.2218
XmaIII.2219
SpcI.2232
SaII.2239
NhcI.2259
BcII.2288
StuI.2307
BspI407.2353

FIG. 52

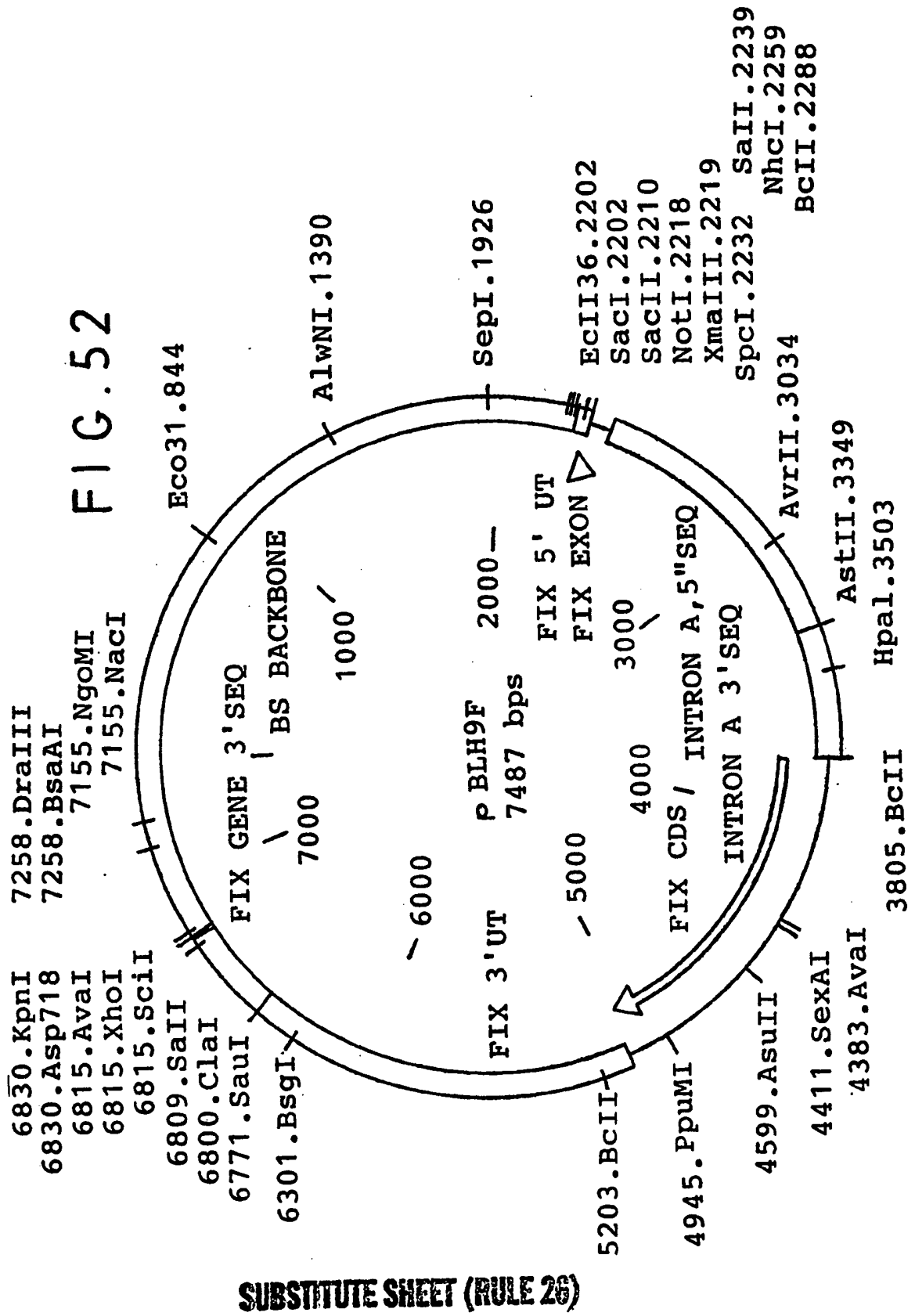

6830.KpnI
6830.Asp718
6815.AvaI
6815.XhoI
6815.SciI
6809.SalI
6800.ClaI
6771.SauI
6301.BsgI
7258.DraIII
7258.BsaAI
7155.NgoMI
7155.NaeI
Eco31.844
AlwNI.1390
SepI.1926
EcII36.2202
SacI.2202
SacII.2210
NotI.2218
XmaIII.2219
SpcI.2232
SalI.2239
NheI.2259
BclI.2288
AvrII.3034
AstII.3349
Hpal.3503
3805.BclI
4383.AvaI
4411.SexAI
4599.AsuII
4945.PpuMI
5203.BclI
FIX GENE 3'SEQ
BS BACKBONE
FIX 3'UT
pBLH9F
7487 bps
FIX 5' UT
FIX EXON
FIX CDS / INTRON A 3'SEQ
INTRON A,5"SEQ
1000
2000
3000
4000
5000
6000
7000

FIG. 53

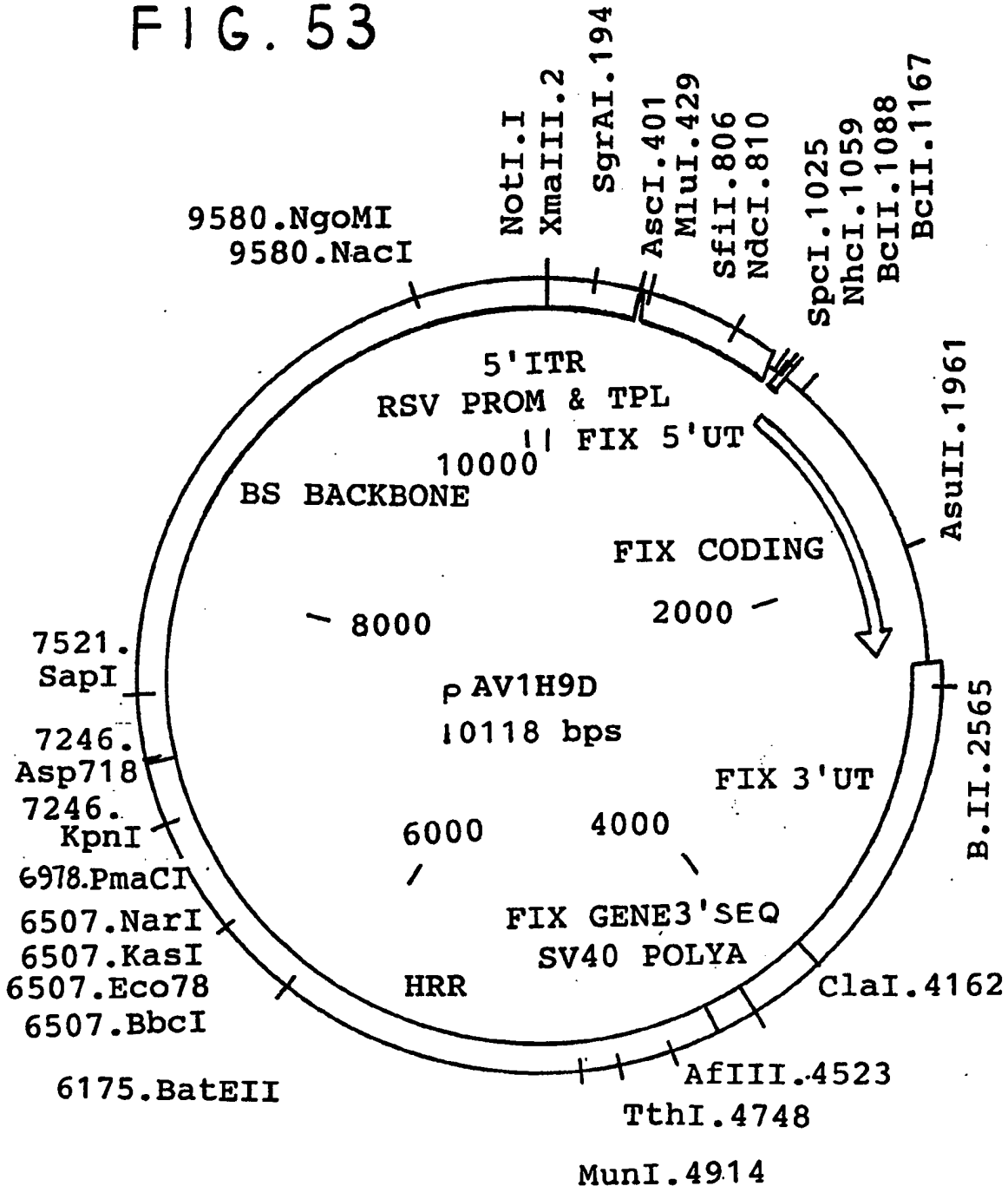
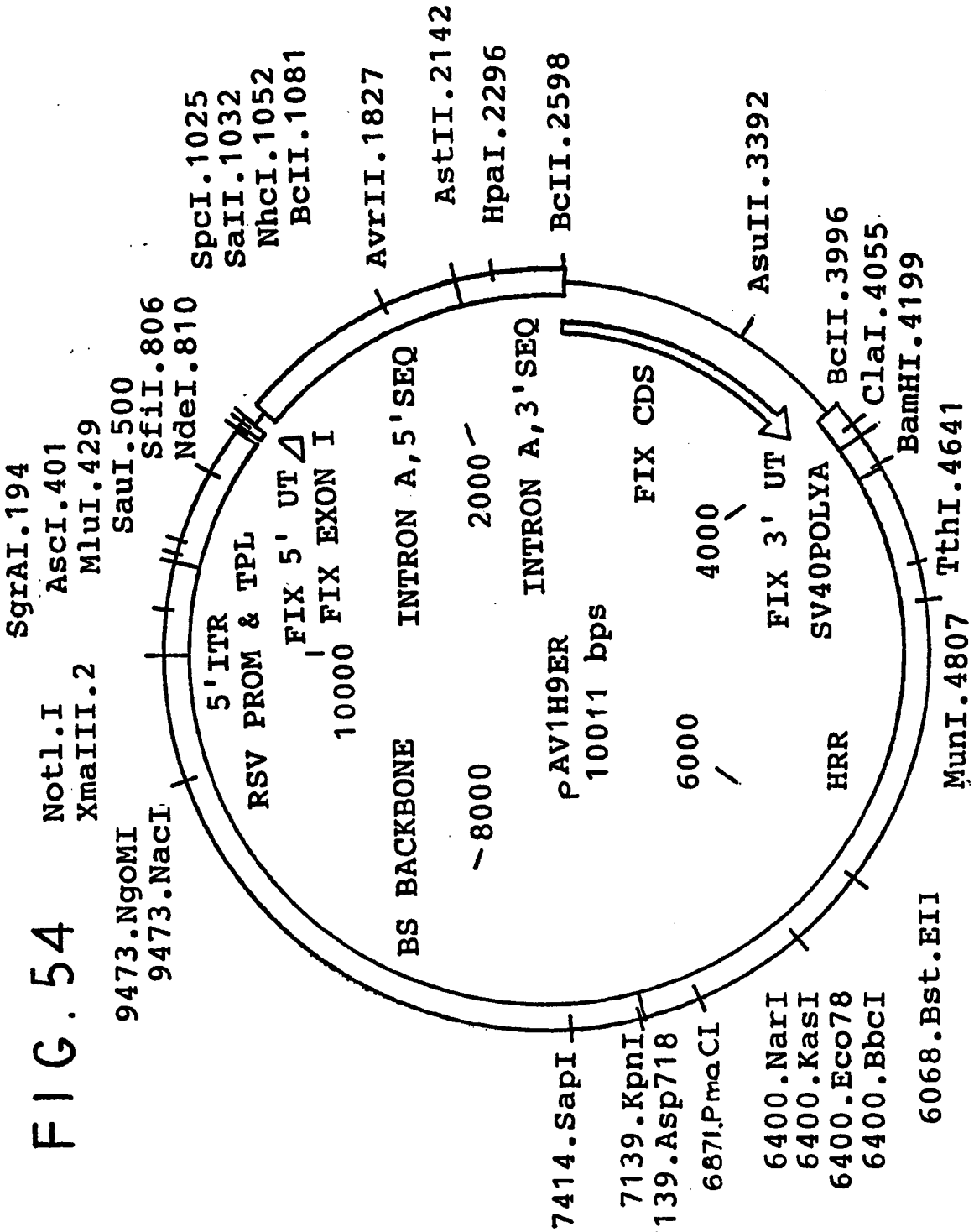

NotI.I
XmaIII.2
SgrAI.194
AscI.401
MluI.429
SfiI.806
NdcI.810
SpcI.1025
NhcI.1059
BclI.1088
BclI.1167
9580.NgoMI
9580.NacI
5'ITR
RSV PROM & TPL
FIX 5'UT
10000
BS BACKBONE
AsuII.1961
FIX CODING
8000
2000
7521.
SapI
pAV1H9D
10118 bps
7246.
Asp718
FIX 3'UT
7246.
KpnI
6000
4000
6978.PmaCI
6507.NarI
FIX GENE3'SEQ
6507.KasI
SV40 POLYA
6507.Eco78
HRR
ClaI.4162
6507.BbcI
6175.BatEII
AflII.4523
TthI.4748
MunI.4914
B.II.2565

FIG. 54


SgrAI.194
AscI.401
MluI.429
SauI.500
SfiI.806
NdeI.810
SpcI.1025
SalI.1032
NhcI.1052
BclI.1081
AvrII.1827
AstII.2142
HpaI.2296
BclI.2598
AsuII.3392
BclI.3996
ClaI.4055
BamHI.4199
TthI.4641
MunI.4807
6068.Bst.EII
6400.NarI
6400.KasI
6400.Eco78
6400.BbcI
6871.PmaCI
7139.Asp718
7139.KpnI
7414.SapI
9473.NgoMI
9473.NacI
NotI.I
XmaIII.2
5'LTR
RSV PROM & TPL
FIX 5' UT
FIX EXON I
INTRON A,5'SEQ
INTRON A,3'SEQ
FIX CDS
FIX 3' UT
SV40POLYA
HRR
BS BACKBONE
pAV1H9ER
10011 bps
10000
8000
2000
4000
6000

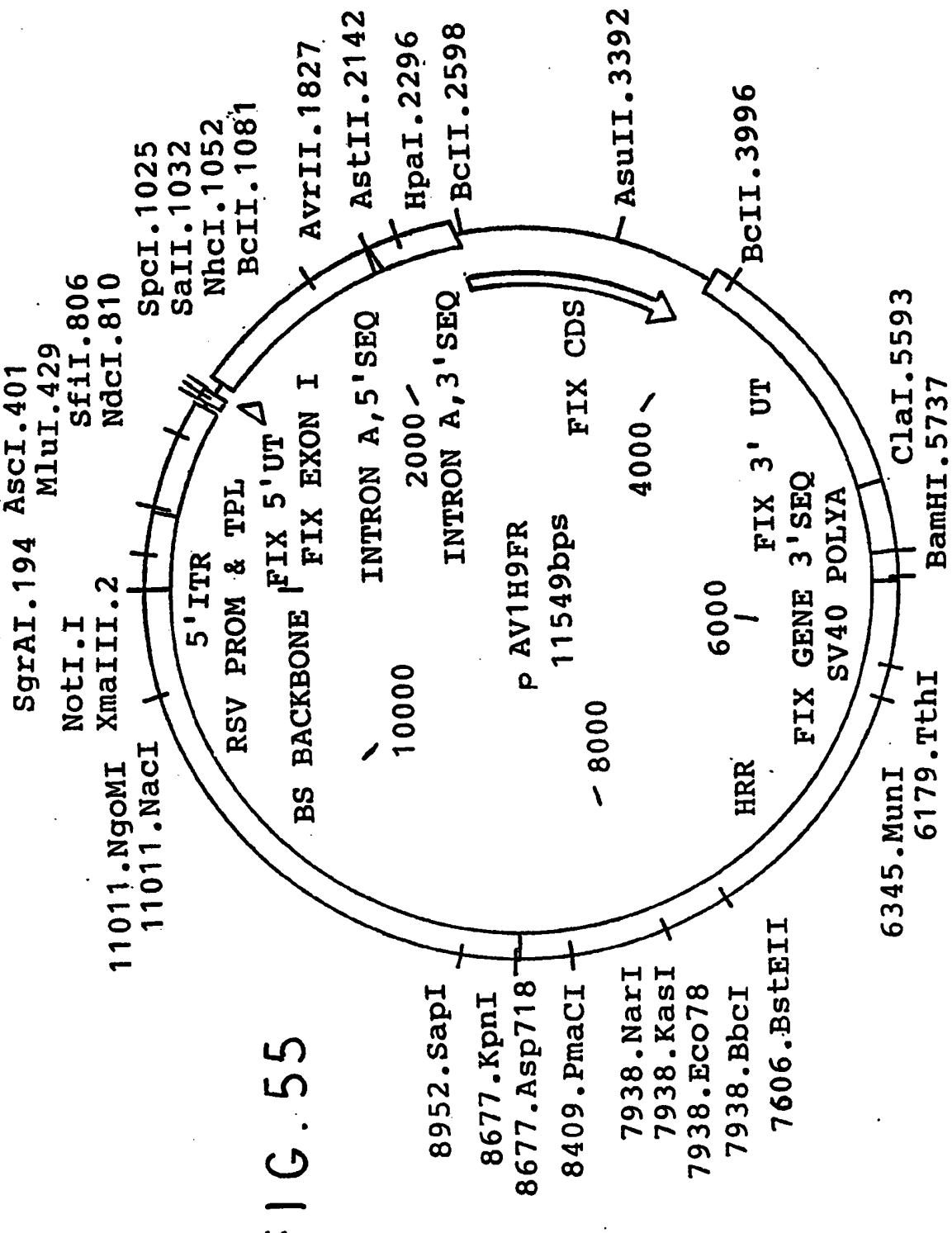
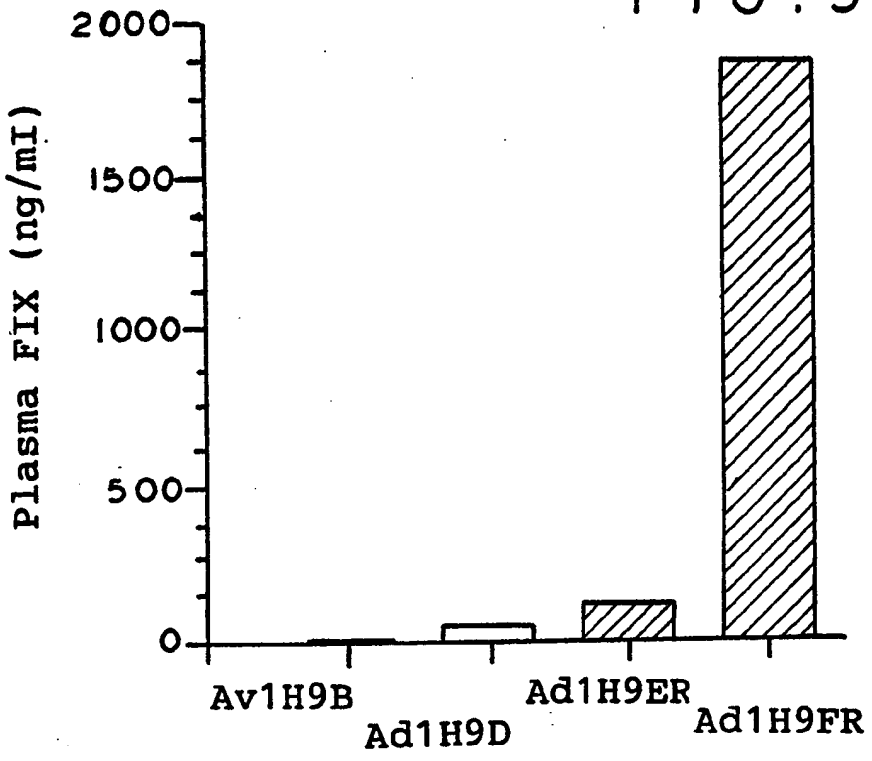

AscI.401
MluI.429
SfiI.806
NdcI.810
SpcI.1025
SalI.1032
NhcI.1052
BclI.1081
AvrII.1827
AstII.2142
HpaI.2296
BclI.2598
AsuII.3392
BclI.3996
ClaI.5593
BamHI.5737
6179.TthI
6345.MunI
7606.BstEII
7938.BbcI
7938.EcoT78
7938.KasI
7938.NarI
8409.PmaCI
8677.Asp718
8677.KpnI
8952.SapI
11011.NacI
11011.NgoMI
XmaIII.2
NotI.I
SgrAI.194
5'ITR
RSV PROM & TPL
BS BACKBONE
FIX 5'UT
FIX EXON I
INTRON A,5'SEQ
INTRON A,3'SEQ
FIX CDS
FIX 3' UT
FIX GENE 3'SEQ
SV40 POLYA
HRR
p AV1H9FR
11549bps
10000
2000
4000
6000
8000

FIG. 55

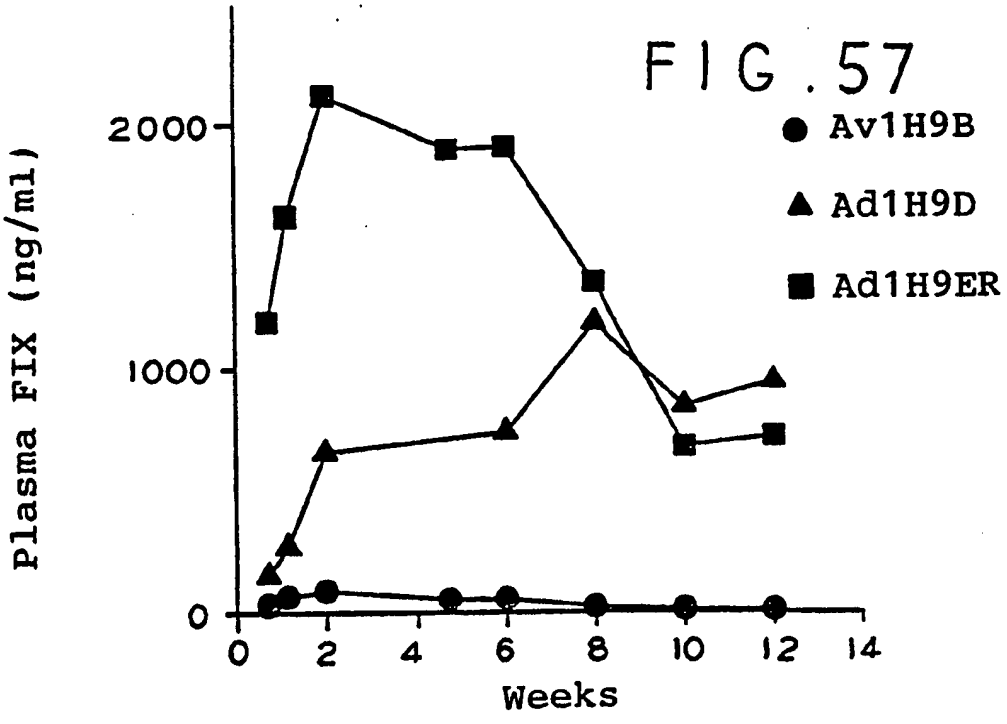
Comparison of Factor IX vectors in Mice
(2×10^8 pfu)

FIG. 56



Comparison of Factor IX Adenovectors in Mice
(1×10^9 pfu)

FIG. 57



Comparison of Factor IX Adenovectors in Mice

(5×10^7 pfu)

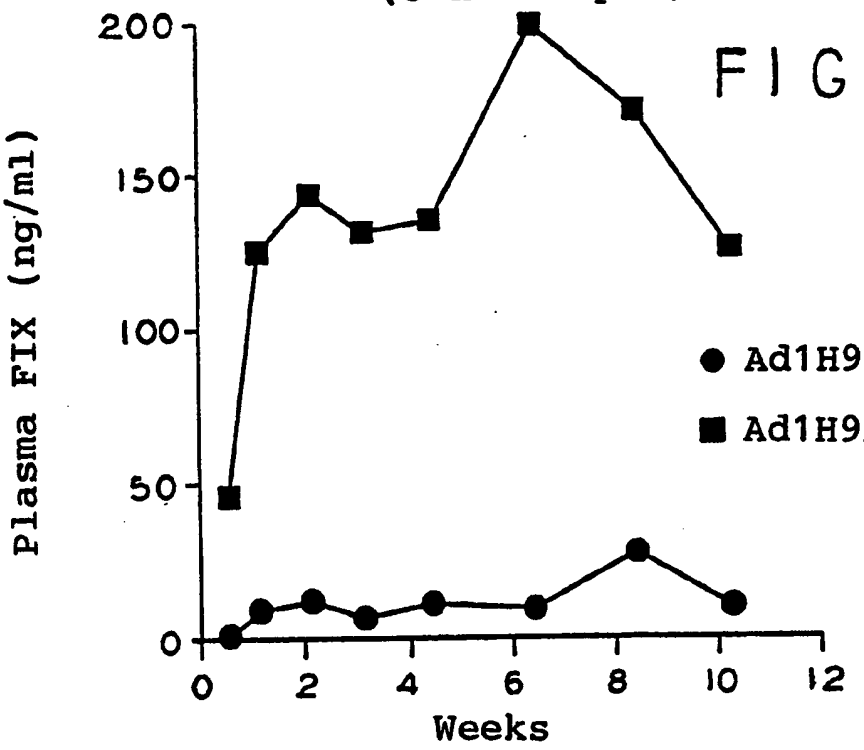
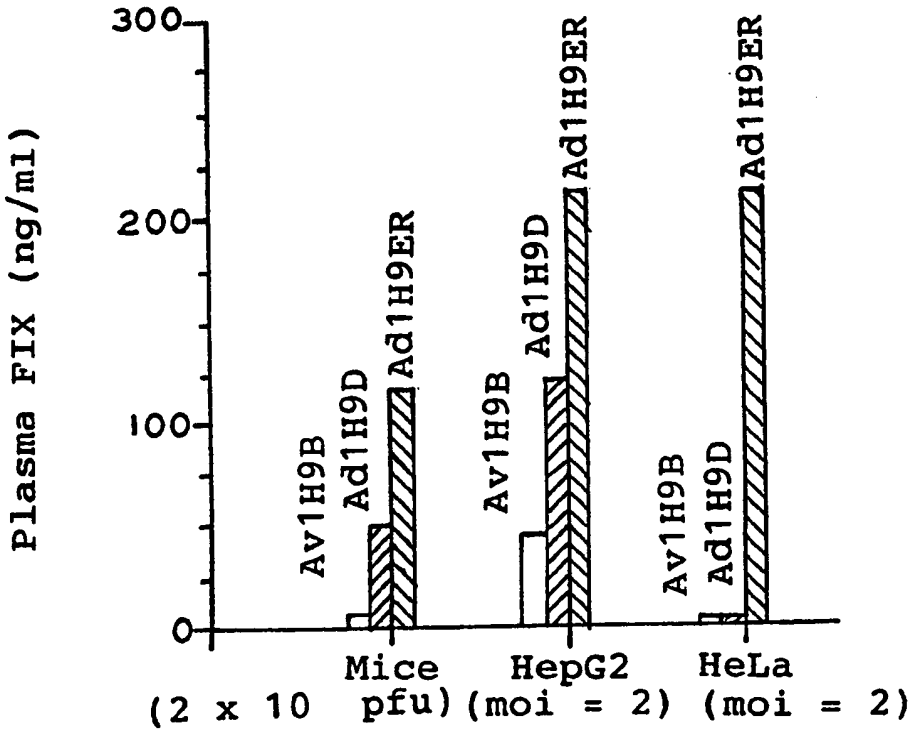


FIG. 58

FIG. 59

Comparison of Factor IX Vectors in vivo and in vitro



INTERNATIONAL SEARCH REPORT

Int ational application No.
PCT/US94/04075

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/86; A61K 48/00
US CL :435/320.1; 424/93A
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1; 424/93A

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; DIALOG DATABASES: BIOSIS PREVIEWS, MEDLINE, WORLD PATENT INDEX, CA SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 93/03769 (CRYSTAL) 04 March 1993, see entire document.	1-20
Y	Blood, Volume 76, Number 2, issued 15 July 1990, Miller, "Progress Toward Human Gene Therapy," pages 271-278, see entire article.	1-20
Y	Bone Marrow Transplantation, Volume 9, Supplement 1, issued 1992, Stratford-Perricaudet et al., "Feasibility of adenovirus-mediated gene transfer *in vitro*," pages 151-152, see entire article.	1-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 23 JULY 1994	Date of mailing of the international search report AUG 09 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOHNNY F. RAILEY II, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Human Gene Therapy, Volume 4, Number 2, issued April 1993, Hoeben et al., "Toward Gene Therapy for Hemophilia A: Long-Term Persistence of Factor VIII-Secreting Fibroblasts after Transplantation into Immunodeficient Mice," pages 179-186, see entire article.	1-20
Y	Proc. Natl. Acad. Sci. USA, Volume 87, issued August 1990, Armentano et al., "Expression of human factor IX in rabbit hepatocytes by retrovirus-mediated gene transfer: Potential for gene therapy of hemophilia B," pages 6141-6145, see entire article.	1-20
Y	Blood, Volume 73, Number 2, issued February 1989, Palmer et al., "Production of Human Factor IX in Animals by Genetically Modified Skin Fibroblasts: Potential Therapy for Hemophilia B," pages 438-445, see entire article.	1-20
Y	Blood, Volume 75, Number 5, issued 01 March 1990, Israel et al., "Retroviral-Mediated Transfer and Amplification of a Functional Human Factor VIII Gene," pages 1074-1080, see entire article.	1-20
Y	Clinical Research, Volume 41, Number 2, issued April 1993, Kopfler et al., "Adenovirus-Mediated Human Apolipoprotein A1 Transfer Increases Circulating HDL Cholesterol in Mice," page 211A, see the abstract.	16-19